UNIVERSIDAD DE SALAMANCA

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DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA

MICOVIRUS ASOCIADOS A LOS HONGOS ENDOFÍTICOS Y ENTOMOPATÓGENOS

Tolypocladium cylindrosporum y *Beauveria bassiana*

Noemí Herrero Asensio

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MICOVIRUS ASOCIADOS A LOS HONGOS ENDOFÍTICOS Y ENTOMOPATÓGENOS *Tolypocladium cylindrosporum* y *Beauveria bassiana*

Memoria presentada por
NOEMÍ HERRERO ASENSIO
para optar al grado de
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Salamanca, a __ de marzo de 2011

DR. ÍÑIGO ZABALGOGEAZCOA GONZÁLEZ, INVESTIGADOR CIENTÍFICO DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC), EN EL INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SALAMANCA,

CERTIFICA

Que la memoria titulada "MICOVIRUS ASOCIADOS A LOS HONGOS ENDOFÍTICOS Y ENTOMOPATÓGENOS *Tolypocladium cylindrosporum* y *Beauveria bassiana"*, presentada por Dña. Noemí Herrero Asensio para optar al grado de Doctora en Biología por la Universidad de Salamanca, ha sido realizada bajo mi dirección, en el Departamento de Estrés Abiótico del Instituto de Recursos Naturales y Agrobiología de Salamanca del Consejo Superior de Investigaciones Científicas (CSIC).

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide y firma el presente certificado en Salamanca, a __ de marzo de 2011.

Fdo. Dr. Iñigo Zabalgogeazcoa González

DR. JOSÉ MARÍA DÍAZ MÍNGUEZ, PROFESOR TITULAR DEL DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA DE LA UNIVERSIDAD DE SALAMANCA,

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Fdo. Dr. José María Díaz Mínguez

DR. ÁNGEL DOMÍNGUEZ OLAVARRI, DIRECTOR DEL DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA DE LA UNIVERSIDAD DE SALAMANCA,

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Y para que así conste, expido y firmo el presente certificado en Salamanca, a __ de marzo de 2011.

Fdo. Dr. Ángel Domínguez Olavarri

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ÍNDICE

1. INTRODUCCIÓN GENERAL

1.1. Consideraciones generales sobre los hongos endofíticos.

Los hongos endofíticos habitan en el interior de las plantas durante todo o parte de su ciclo de vida sin causarles síntomas aparentes. Se conoce de la existencia de este tipo de hongos desde finales del siglo XIX, cuando Guerin (1898) y Vogl (1898) descubrieron hongos en el interior de semillas de cizaña (*Lolium temulentum*). No obstante, estos hongos no recibieron especial atención hasta bien entrado el siglo XX. Fue precisamente en 1977 que se impulsó el estudio de los endofitos, cuando Charles Bacon y sus colaboradores demostraron que las toxicosis observadas en ganado vacuno estadounidense, eran debidas al consumo de plantas de *Lolium arundinaceum* (=*Festuca arundinacea*), cuyas hojas y tallos estaban infectados sistémicamente por el hongo *Neotyphodium coenophialum*, sin que las plantas mostrasen ningún síntoma (Bacon *et al*., 1977). Más tarde se descubrió que las toxicosis eran debidas a la producción de alcaloides por parte de *N. coenophialum*. Era por lo tanto el endofito el agente causal de los trastornos del ganado. De este modo, la presencia de un hongo productor de alcaloides resultaba beneficiosa para la planta hospedadora, puesto que la protegía del estrés biótico derivado del herbivorismo (Schardl *et al*., 2004).

Hoy en día, las especies del género *Neotyphodium* y de su teleomorfo *Epichloë* constituyen el grupo más conocido y estudiado dentro de los hongos endofíticos, y son conocidos en su conjunto como hongos endofíticos sistémicos de gramíneas. Estos hongos pertenecientes a la familia *Clavicipitaceae* representan solo una pequeña fracción de los hongos asociados a gramíneas, pues algunas de éstas, como *Dactylis glomerata* u *Holcus lanatus* (Sánchez Márquez *et al*., 2007; 2010) pueden ser hospedadores de más de cien especies diferentes de hongos endofíticos. Son precisamente los hongos aislados de estas y otras gramíneas, los que se utilizaron para el análisis de incidencia de micovirus en el grupo de hongos endofitos no sistémicos de gramíneas (Sánchez Márquez *et al.,* 2011). Por otra parte, los endofitos son ubicuos en el reino vegetal, en los últimos años se han llevado a cabo numerosos estudios de incidencia de endofitos, y todos ellos parecen apuntar a que la mayoría de las especies vegetales presentes tanto en comunidades naturales como antropogénicas, extendidas desde el ártico a los trópicos, son hospedadoras de estos hongos (Arnold, 2007; Stone *et al*., 2004).

Los hongos endofíticos colonizan normalmente el espacio intercelular de la planta a la que infectan, localizándose tanto en las partes aéreas como en las subterráneas, pudiendo ser específicos de un órgano. De este modo, pueden dar lugar a infecciones sistémicas,

como ocurre en el caso de *Neotyphodium* y *Epichloë,* o a infecciones muy localizadas, como en el caso de los endofitos no sistémicos (Rodríguez *et al*., 2009). Por otro lado, el rango de hospedadores de las diferentes especies de endofitos también es variable, habiendo especies con un único hospedador y especies muy generalistas (Sánchez Márquez *et al.,* 2011; Stone *et al*., 2004).

El mecanismo de dispersión predominante entre los endofitos es la transmisión horizontal, como lo indica el hecho de que la mayoría de las semillas estén libres de endofitos, y que la incidencia de endofitos sea mayor en las plantas a medida que aumenta su edad (Arnold y Herre, 2003; Sánchez Márquez *et al.,* 2011). Ahora bien, varias especies de los géneros *Neotyphodium* y *Epichloë* tienen como medio de dispersión la transmisión vertical a las semillas (Schardl *et al*., 2004).

La relación entre las plantas y los hongos parece datar de muy antiguo. El registro fósil indica que estas asociaciones pudieron comenzar hace más de 400 millones de años, en la era Paleozoica (Bacon y Hill, 1995; Oliver, 1903; Osborn, 1909). Este hecho revela la importancia de estas asociaciones planta-hongo, pues en términos evolutivos los hongos endofíticos podrían llevar ligados a las plantas desde que éstas dieron el salto a tierra y la colonizaron, desempeñando desde entonces un papel crucial en la evolución de las plantas en el medio terrestre. Por este motivo, se cree que los hongos endofíticos mantienen en ocasiones asociaciones mutualistas con sus hospedadores, establecidas posiblemente gracias a un proceso de coevolución (Hyde y Soytong, 2008). Un ejemplo de ello son las establecidas por especies del género *Epichloë*, que como ya comentamos, producen una serie de alcaloides que confieren a las plantas que infectan un aumento de la resistencia al herbivorismo (Schardl *et al*., 2004). Otras especies de endofitos son conocidas por su capacidad de mejorar la resistencia de sus hospedadores a ciertas enfermedades, compitiendo directamente con el patógeno por el espacio y los recursos, o incluso produciendo compuestos nocivos para estos patógenos (Zabalgogeazcoa, 2008). Como aplicación, algunos hongos endofíticos cuando son inoculados artificialmente pueden conferir a sus hospedadores resistencia a estreses abióticos tales como la falta de agua, la salinidad, o las altas temperaturas, e incluso pueden producir un aumento en la biomasa de las plantas a las que colonizan (Rodríguez *et al*., 2009; Waller *et al*. 2005). Ahora bien, para la mayoría de especies de endofitos descritas hasta el momento, los efectos de la asociación simbiótica con su hospedador son desconocidos.

1.2. Hongos endofíticos y entomopatógenos, dos grupos aparentemente unidos.

Los hongos entomopatógenos parasitan insectos causándoles graves daños que pueden incluso ocasionar su muerte. Existen estudios filogenéticos que apuntan a que el origen de algunos hongos endofíticos podía estar en un ancestro entomopatógeno. Miembros de la familia *Clavicipitaceae* como *Epichloë* o *Neotyphodium*, a los que nos hemos referido en el apartado anterior por su naturaleza endofítica, podrían haber sido en sus orígenes parásitos de insectos. Se cree que estos hongos consiguieron el acceso a los nutrientes vegetales a través del estilete de sus hospedadores o de las heridas que éstos ocasionaban en las plantas, evolucionando así hacia formas biotrofas vegetales (Rodríguez *et al.,* 2009). De este modo, estos hongos llegarían a desarrollar capacidad endofítica. Esta idea queda reforzada por el hecho de que los endofitos *Epichloë* y *Neotyphodium* no producen enzimas o toxinas para matar o degradar tejidos vegetales, lo cual podría facilitar la colonización de las plantas al no favorecer mecanismos defensivos en éstas. Además, la producción por parte de estos hongos de compuestos biológicamente activos nocivos para animales, especialmente insectos, es concordante con la idea de un ancestro entomopatógeno (Rodríguez *et al*., 2009; Spatafora *et al*., 2007). Se ha visto que este grupo de hongos produce efectos negativos a través de los alcaloides que producen en sus plantas hospedadoras, en más de 40 especies de insectos, estableciéndose así una relación mutualista entre la planta y el hongo (Vega *et al.,* 2008).

Otros hongos pertenecientes a las familias *Cordycipitaceae* y *Ophiocordycipitaceae* (antiguos miembros de la familia *Clavicipitaceae* recientemente reclasificados) que incluyen géneros de hongos considerados tradicionalmente entomopatógenos, han sido también descritos como endofitos naturales, parasitando plantas de naturaleza muy diversa, que van desde gramíneas, como el arroz o el maíz, hasta plantas dicotiledóneas, como el cacao o el café. Estos géneros de entomopatógenos endofíticos son *Beauveria*, *Verticillium*, *Paecilomyces*, *Cladosporium*, *Metarhizium, Lecanicillium* o *Tolypocladium* (Sánchez Márquez *et al.*, 2011; Vega, 2008).

Además de un estudio de la incidencia de micovirus en hongos endofíticos de gramíneas, en este trabajo también se analizarán los virus asociados a dos hongos entomopatógenos también aislados como endofitos, *Beauveria bassiana* y *Tolypocladium cylindrosporum* (Sánchez Márquez *et al.*, 2007; 2010). Adicionalmente, también se estudiará la capacidad endofítica de *T. cylindrosporum*. Por ello en este apartado dedicado

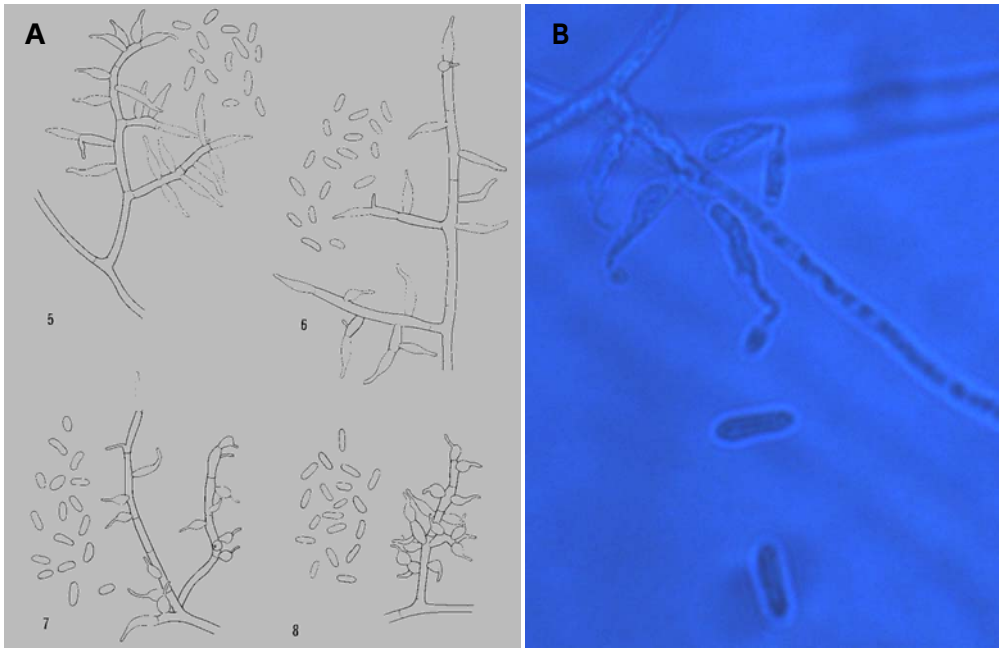
a hongos endofíticos y entomopatógenos se hará un pequeño repaso a las características generales de estas dos especies.

1.2.1. *Tolypocladium cylindrosporum*

El género *Tolypocladium* fue establecido por Gams en el año 1971 basándose para ello en más de 10 aislados fúngicos procedentes de suelos y céspedes de diferentes partes de Europa. En un principio, Gams describió tres especies en este nuevo género: *T. inflatum*, *T. cylindrosporum* y *T. geodes* (Weiser y Pillai, 1981). Barron atribuyó a este género otras dos especies aisladas de rotíferos *T. parasiticum* y *T. trigonosporum* (Barron, 1980; 1981). De este modo, el número de especies que fueron incorporándose al género *Tolypocladium* fue aumentando durante la década de los 80, hasta llegar al total de 15 especies que en la actualidad lo conforman (Bisset, 1983; Samson y Soares, 1984; Weiser *et al.,* 1991).

El género *Tolypocladium* ha sido recientemente clasificado en la familia *Ophiocordycipitaceae* (Sung *et al.,* 2007), dentro del orden *Hypocreales*. Al igual que el resto de especies que componen el género, *T. cylindrosporum* se caracteriza por producir colonias de aspecto algodonoso de crecimiento relativamente rápido, alcanzando 7-20 mm de diámetro en 10 días a 22 ºC. Su coloración suele ser blanca al principio y a veces torna a color crema en zonas donde la conidiación es abundante. El reverso de las colonias va de blanco a amarillo pálido o naranja. Aunque no suele producir exudados, a veces puede dar lugar a pequeñas gotitas incoloras. En cuanto a la morfología de las fiálidas, éstas presentan la parte inferior más o menos globosa y la superior se estrecha para dar lugar a un cuello cilíndrico largo (Fig. 1). Los conidios también son muy característicos, de hecho su morfología da el nombre a la especie, ya que tienen forma de cortos cilindros (4.6 x 1.6 μm) con ambos extremos totalmente redondeados. Suelen agregarse en pequeñas cabezas en las puntas de las fiálidas, de hecho, en cultivos más viejos estos conidios coalescen para dar lugar a masas extensivas y viscosas (Bissett, 1983).

Fig. 1. A. Representación gráfica de las características morfológicas de *T. cylindrosporum* (Bisset, 1983). **B.** Fotografía de hifas, fiálidas y esporas de la cepa 11 de *T. cylindrosporum*.



La mayoría de las especies de este género habitan en el suelo. *Tolypocladium* tiene afinidad por suelos de zonas frías o de hábitats sujetos a fuertes oscilaciones térmicas diarias o estacionales. La temperatura óptima de crecimiento del género en agar de dextrosa y patata (PDA) es para todas las especies en torno a los 20 ºC, y la mínima se sitúa en los 5 ºC, todas las especies muestran tasas mínimas de crecimiento a 30 ºC, hasta el punto que *T. geodes* deja de germinar o crecer a esta temperatura (Bissett, 1983).

Aunque nos hemos referido a este género como característico de suelos, poco después de su descubrimiento en 1971, *T. cylindrosporum* fue aislado en California de un mosquito, *Aedes sierrensis* y después en Nueva Zelanda de *Aedes australis*. De hecho, se han encontrado alrededor de 19 especies de mosquito susceptibles a este hongo, incluyendo géneros como *Aedes*, *Culex*, *Culiseta* o *Anopheles* (Scholte *et al.,* 2004), todos ellos de gran interés por la relación que guardan con la transmisión del *Plasmodium* que produce la malaria. También se han encontrado otros artrópodos susceptibles a *T. cylindrosporum,* entre los que destacan otros dípteros terrestres y acuáticos, lepidópteros, algunos crustáceos como *Daphnia carinata* o *Trigriopus* sp. (Lam *et al.,* 1988) e incluso garrapatas de la familia *Argasidae* (Herrero *et al.,* 2011; Zabalgogeazcoa *et al*., 2008).

El proceso de infección de *Tolypocladium* comienza con la adhesión de las esporas del hongo a la cutícula de sus hospedadores. La zona en que comienza este proceso de infección es diferente según el estado de desarrollo de los hospedadores. Estas esporas germinarán y atravesarán la cutícula, comenzando así la colonización del hemocele de la víctima. Los conidios también pueden llegar a colonizar el espacio interior del hospedador utilizando para su entrada cavidades naturales. Así, los conidios germinan en las proximidades de la cavidad bucal y anal, ramificándose hasta alcanzar el hemocele (Goettel, 1988). Durante el proceso de colonización, *T. cylindrosporum* produce blastosporas en el hemocele, que germinarán y atravesarán de nuevo la cutícula del hospedador para seguir desarrollándose en el exterior. La esporulación del hongo en la superficie del hospedador va a servir como fuente de inóculo para otros posibles hospedadores (Goettel, 1988). Durante la infección *Tolypocladium* produce una serie de sustancias insecticidas que van a ayudar a terminar con el hospedador. Estas sustancias son diversas y entre ellas destacan las efrapeptinas y la tolypina (Bandani *et al.*, 2000; Weiser y Matha, 1988). De este modo, *T. cylindrosporum* se convierte en un excelente candidato a arma de control biológico, más aún desde que se conoce su naturaleza endofítica, al haber sido aislado como tal de las gramíneas *Holcus lanatus* y *Festuca rubra* (Sánchez Márquez *et al.*, 2010).

Por otro lado, el género *Tolypocladium* es bien conocido por su importancia biotecnológica en la producción de ciclosporinas (Aarnio y Agathos, 1989). La ciclosporina A es un péptido cíclico que presenta una fuerte y selectiva actividad inmunosupresiva, habiendo llegado a convertirse en un agente imprescindible en la cirugía de trasplantes dado que permite superar las barreras de la histocompatibilidad. Este compuesto también se ha empleado en el tratamiento de enfermedades parasíticas o autoinmunes, así como en quimioterapia (Reed y Thomas, 2008) *T. inflatum* es la especie que produce las mayores cantidades de ciclosporina, aunque *T. cylindrosporum* también produce esta preciada sustancia (Aarnio y Agathos, 1989).

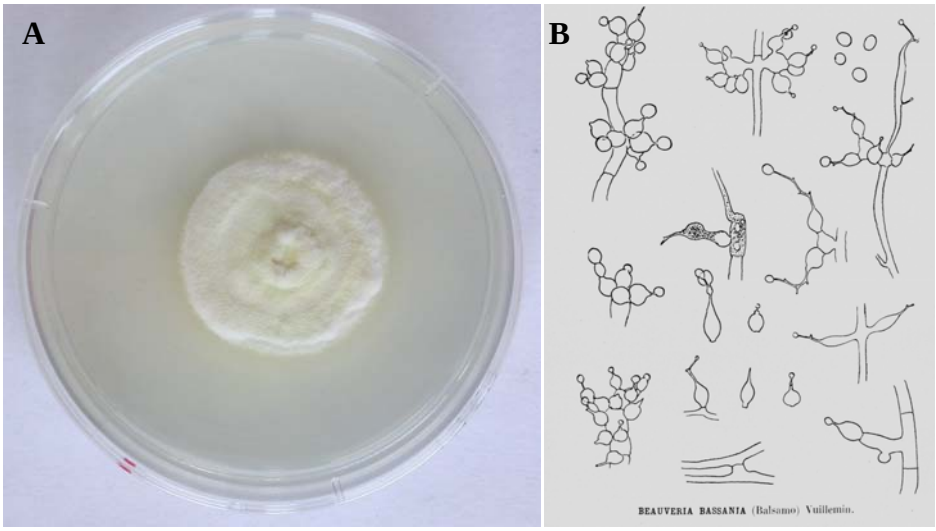
1.2.2. *Beauveria bassiana*

En 1835, Agostino Bassi fue el primero en describir a *Beauveria* como agente causal del *mal del segno*, también conocido en Italia como *calcinaccio* o *cannellino* y como *muscardino blanco* en Francia. Este hongo fue el causante de la devastadora epizoosis que produjo importantes pérdidas económicas en la industria de la seda del sur de

Europa, entre los siglos XVIII y XIX. La epidemia diezmó las poblaciones de larvas de gusanos de seda, y por ello se desarrollaron diversos estudios en este ámbito. En esta línea, Bassi se dedicó al estudio de *Beauveria,* y con ello, fue el primero en demostrar que los microbios pueden actuar como patógenos contagiosos de animales, una importante aportación a la teoría del germen de la enfermedad (Porter, 1973).

El primer reconocimiento taxonómico del hongo muscardino fue propuesto por Balsamo-Crivelli, quien en honor a Bassi, denominó al hongo *Botrytis bassiana*. El género *Beauveria*, en cambio, no fue descrito formalmente hasta 1912 por Vuillemin, y actualmente se engloba en la familia *Cordycipitaceae,* dentro del orden Hypocreales. *B. bassiana* presenta un aspecto algodonoso y tiene una coloración blanca que en ocasiones torna a crema o amarillo anaranjado. Presenta fiálidas cortas y globosas en la base y estrechas en su parte superior, agrupadas normalmente en verticilos (Fig. 2). Cada una de estas fiálidas porta un conidio en forma de balón (Vuillemin, 1912). Ahora bien, el reconocimiento morfológico de las distintas especies que engloba el género *Beauveria* es bastante complicado, puesto que las características morfológicas son muy similares entre especies. Actualmente la identificación de los miembros de este género suele hacerse molecularmente a través de la secuencia de espaciadores internos transcritos (ITSs) en los operones de RNA ribosómico, y de factores de elongación 1-alfa (EF1-α) (Rehner y Buckley, 2005).

Fig. 2. A. Cultivo de *Beauveria bassiana* en agar de dextrosa y patata. **B.** Representación gráfica de las características morfológicas de las fiálidas y conidios de *B. bassiana* (Vuillemin, 1912).



Beauveria es un género muy cosmopolita que engloba a hifomicetes haploides que habitan en el suelo de forma natural. *B. bassiana* es el hongo entomopatógeno con más representación en los suelos españoles, tanto cultivados como naturales, de hecho existe un estudio sobre la distribución de este hongo en la península y como las características del terreno afectan a dicha distribución. Según este estudio, *B. bassiana* tiene predilección por suelos con bajo contenido en materia orgánica y preferiblemente arcillosos, y suele localizarse por lo general en latitudes bajas y altitudes por debajo de los 700 m (Quesada-Moraga *et al.*, 2007). Es preciso resaltar, que algunos de los aislados de *B. bassiana* derivados de este estudio, sirvieron de material de partida para el estudio de incidencia de micovirus en dicha especie desarrollado en este trabajo.

Este género es muy considerado en nuestros días debido a su papel como patógeno de insectos y a su capacidad para producir metabolitos de interés médico. El modo de infección de *B. bassiana* es similar al de *T. cylindrosporum* y conlleva la adhesión de las esporas a la cutícula del insecto, seguido de la formación de un tubo germinativo que a través de acciones enzimáticas y mecánicas logra penetrar la cutícula. Una vez en el hemocele, el crecimiento de las hifas causa daños en los tejidos del insecto y absorbe sus nutrientes. Dentro del insecto *B. bassiana* puede producir metabolitos secundarios, cuya actividad insecticida, permite al hongo acabar con el insecto de una forma más rápida (Clarkson y Charnley, 1996). Algunas de las sustancias con propiedades insecticidas producidas por el hongo son péptidos cíclicos, como la beauvericina, las eniatinas, basianólidos, o incluso compuestos de interés medicinal como las ciclosporinas o la oosporina (Fuguet *et al.*, 2004). Tras la invasión del hemocele, el hongo es capaz de volver a atravesar la cutícula del hospedador y salir al exterior, donde puede continuar desarrollándose saprofiticamente sobre los cadáveres en los que esporula, convirtiéndolos en nuevos focos de diseminación de esporas de *Beauveria* (Meyling y Eilenberg, 2007). Es preciso resaltar que *B. bassiana* es el hongo entomopatógeno que ha sido sujeto de más estudios de cara a su aplicación comercial como biocontrolador. De hecho, son varias las formulaciones de esporas que actualmente se comercializan con este objeto (Kaufman *et al.,* 2005).

Beauveria bassiana y su teleomorfo *Cordyceps bassiana*, han sido el centro de múltiples estudios como endofitos, de hecho, han sido descritos infectando de forma natural a numerosos hospedadores, desde gramíneas hasta dicotiledóneas como patata, algodón ó cacao. Además, se han realizado múltiples ensayos de inoculación artificial de

B. bassiana en diferentes especies vegetales de interés comercial, como cacao, amapola, banana o café, con el propósito de introducir a los entomopatógenos endofíticos como una alternativa al uso de insecticidas químicos (Quesada-Moraga *et al.,* 2006a; Vega *et al*., 2008). Por otra parte, *B. bassiana* también parece poseer una importante actividad fungicida y bactericida, pues como ya hemos apuntado, el hongo es productor de una enorme batería de metabolitos bioactivos. En esta línea *B. bassiana* podría ser capaz de proteger del ataque de microorganismos fitopatógenos a la planta en la que se encuentra como endofito (Ownley *et al.,* 2010). De este modo, el hábitat endofítico de *B. bassiana* podría proporcionar beneficios tanto a la planta como al hongo, lo que se viene conociendo como la "*bodyguard hypothesis*". Según esta hipótesis, las plantas favorecerían la retención de enemigos naturales de plagas herbívoras o de microorganismos fitopatógenos para protegerse del ataque de éstos. Al mismo tiempo, el hongo quedaría protegido de diversos estreses ambientales, y obtendría diversos nutrientes y exudados producidos por la planta hospedadora (Elliot *et al.,* 2000; Ownley *et al.,* 2010).

El repaso realizado a estas dos especies entomopatógenas que serán sujeto de estudio en este trabajo, pone de manifiesto el paralelismo existente entre sus ciclos de vida. De este modo, los dos hongos que parecen presentarse como hongos típicos del suelo, en algún momento, seguramente vía conidios, van a ser capaces de parasitar insectos y desarrollar parte de su ciclo vital en el interior de éstos. Ahora bien, también poseen la capacidad de desarrollar un periodo de su ciclo de vida en el interior de una planta, como endofitos. Así, este hábitat, podría servir como una especie de reservorio que proteja a estas especies de la adversidad, para poder después colonizar los otros dos medios afines a estos hongos y así cerrar un ciclo. De este modo, los otros dos hábitats en que los hongos entomopatógenos son capaces de desarrollarse, suelo y en especial insectos, podrían ser más adecuados para la reproducción, crecimiento y dispersión de estos hongos (Meyling y Eilenberg, 2007; Vega *et al.*, 2008).

1.3. Características generales de los micovirus y su paralelismo con los hongos endofíticos.

Los virus de hongos constituyen una parcela bastante inexplorada dentro de la virología. De hecho, su historia es bastante reciente y la primera descripción de un

micovirus data de 1962, año en que Hollings descubrió partículas virales infectando champiñones (Hollings, 1962). No obstante, a pesar de no haber recibido tanta atención como los virus de plantas o animales, han sido muchos los micovirus descritos en estos casi 50 años de estudio. Se han descrito virus en los cuatro phyla de los hongos, Chytridiomycota, Zygomycota, Ascomycota y Basidiomycota, y también en oomicetos patógenos de plantas como *Phytophthora* y *Phytium*.

A diferencia de los virus animales o vegetales, que normalmente están asociados a enfermedades, muchos de los micovirus conocidos no son causantes de síntomas aparentes en su hospedador, de hecho, según refleja la literatura, la mayoría de los hongos infectados no son fenotípicamente diferentes de los que no lo están, al menos en las condiciones en que son observados en el laboratorio (Ghabrial, 1998; Ghabrial y Suzuki, 2008). Este tipo de asociación virus-hongo recuerda bastante a las ya comentadas entre hongos y plantas, en las que los hongos endofíticos no causan una sintomatología obvia a la planta a la que infectan. De este modo, el desarrollo de nuevas formas de abordar el estudio de las relaciones virus-hongo, quizás ayude en un futuro a entender la naturaleza de estas relaciones simbióticas. No obstante, al igual que ocurre con los hongos endofíticos, existen algunos casos en los que un micovirus produce un efecto observable en su hospedador, causando en ocasiones hipovirulencia, es decir, una disminución en la virulencia de su hospedador hacia la planta a la parasita. Este fenómeno está muy bien documentado para *Cryphonectria parasitica,* el hongo causante del chancro del castaño (Deng *et al*., 2007). También existe un caso en el que un micovirus causa enfermedad a su hospedador. Así, la enfermedad de La France del champiñón, que tantas pérdidas ocasiona en el sector productor de este hongo, tiene su agente causal en el virus LIV (La France isometric virus) (Romaine y Goodin, 2002). Sin embargo, otros micovirus pueden ser beneficiosos, siendo este el caso de la relación mutualista entre el micovirus CThTV (*Curvularia* termal tolerance virus), el hongo endofítico *Curvularia protuberata* y su planta hospedadora *Dichanthelium lanuginosum*, en la que el virus proporciona termotolerancia al conjunto simbiótico mutualista (Marquez *et al.,* 2007).

En cuanto a algunas características bioquímicas de estos virus, es importante señalar que la mayoría de los micovirus conocidos poseen genomas de RNA bicatenario (dsRNA), de hecho, la presencia de elementos de dsRNA en un hongo suele relacionarse con una infección vírica y la presencia de dsRNA suele usarse en diagnosis (Zabalgogeazcoa *et al*., 1995). Ahora bien, también se han descrito varios micovirus con

genomas de RNA monocatenario (ssRNA). De hecho, algunos micovirus que se pensaba que poseían genomas de dsRNA como los hypovirus, barnavirus y narnavirus poseen genomas de ssRNA. Así, el dsRNA encontrado en los extractos de micelio de estos hongos representaría estados intermedios de replicación de un virus de ssRNA (Esteban y Fujimura, 2003; Nuss, 2005; Revill, 2010). Es preciso señalar que también existen dos familias de micovirus, *Pseudoviridae* y *Metaviridae,* que aunque poseen genomas de RNA necesitan un intermediario de DNA para su replicación (ssRNA-RT). Los genomas de DNA no parecen ser comunes en este grupo de virus, y hasta el momento sólo se conoce un micovirus con genoma de DNA monocatenario (ssDNA) y circular, SsHADV-1 (*Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1) (Yu *et al.,* 2010).

Atendiendo a todo lo mencionado sobre la naturaleza del genoma de los micovirus, es preciso resaltar que la clasificación de micovirus propuesta por el International Committee for the Taxonomy of Viruses (ICTV) se basa en las características del genoma de los micovirus para su clasificación taxonómica. Según esta clasificación en la actualidad se conocen nueve familias y doce géneros de virus de hongos (Tabla 1).

Tabla 1. Clasificación taxonómica de micovirus según el ICTV Master Species List 2009 (http://talk.ictvonline.org/files/ictv_documents/m/msl/1231.aspx).

Genoma	Familia	Género
ss(+)RNA	*Barnaviridae*	*Barnavirus*
	Hypoviridae	*Hipovirus*
	Narnaviridae	*Mitovirus*
		Narnavirus
ss(+) RNA-RT	*Pseudoviridae*	*Hemivirus*
		Pseudovirus
	Metaviridae	*Metavirus*
dsRNA	*Chrysoviridae*	*Chrysovirus*
	Partitiviridae	*Partitivirus*
	Reoviridae	*Mycoreovirus*
	Totiviridae	*Totivirus*
		Victorivirus

Los micovirus poseen genomas bastante más pequeños y simples que los virus animales o vegetales, de hecho, existe una familia, *Narnaviridae,* cuyos miembros poseen genomas de 3 kbp que codifican una RNA polimerasa dependiente de RNA (RdRp) que es capaz de replicarles a ellos mismos (Esteban y Fujimura, 2003). Los mayores genomas los vamos a encontrar en la familia *Reoviridae,* pero no van a superar los 23 kbp.

Además de su simplicidad, otra característica que también parece ser común a todos los genomas de virus de hongos, es su linearidad (SsHADV-1 constituye una excepción, presentando genoma circular (Yu *et al.,* 2010). No obstante, su organización puede variar desde genomas monopartitos, como en los totivirus, narnavirus ó barnavirus, a genomas segmentados. Así, los partitivirus se caracterizan por genomas bipartitos, los chrysovirus por tetrapartitos y los micoreovirus pueden tener genomas compuestos hasta por 12 segmentos de dsRNA (Fauquet *et al*., 2005; Hillman, 2010). Generalmente estos genomas van a estar encapsidados en partículas isométricas y proteicas. Si bien, los miembros de la familia *Hypoviridae*, protegen sus genomas en el interior de vesículas membranosas originadas por el hospedador y los *Narnaviridae* carecen de toda envoltura, presentando genomas desnudos.

El modo de transmisión de los micovirus es muy distintivo de este grupo de virus. Así, a diferencia de los virus vegetales o animales, que pueden permanecer fuera de las células del huésped y transmitirse por vectores u otros medios, los micovirus no tienen una fase extracelular. El ciclo de vida de estos virus parece estar limitado a las células fúngicas, y su transmisión a nuevos hospedadores depende de eventos de anastomosis en los que hay intercambios citoplasmáticos entre diferentes aislados compatibles (Pearson *et al.,* 2009).
Por otro lado, la transmisión vertical de los virus a las esporas asexuales constituye un medio común de dispersión de los micovirus, en cambio, en algunas especies, normalmente ascomicetes, parecen existir barreras para la transmisión de virus durante la reproducción sexual y la formación de esporas sexuales (Romo *et al.,* 2007), aunque en otras especies, que suelen coincidir con representantes del grupo de los basidiomicetes, la transmisión viral a las esporas sexuales puede ser muy elevada (Pearson *et al.,* 2009). El hecho de que la transmisión de micovirus esté limitada a mecanismos intracelulares, hace que el movimiento de virus entre distintas especies sea poco frecuente, ya que la transmisión de virus por fusión de hifas, requiere que esta tenga lugar entre grupos de hongos compatibles de la misma especie. No obstante, existen casos en los que bajo condiciones de estrés se ha observado la transmisión de micovirus entre aislados no

compatibles, e incluso entre hongos de distintas especies (Charlton y Cubeta, 2007; Melzer *et al.,* 2002).

En cuanto al origen de los micovirus, una de las hipótesis sostiene que las asociaciones entre los micovirus y sus hospedadores deben ser muy antiguas, defendiendo una hipótesis coevolutiva. Así, un largo periodo de coevolución podría explicar la existencia de fenotipos asintomáticos en muchas infecciones por micovirus (Pearson *et al.,* 2009). El caso de las infecciones sintomáticas por micovirus en el hongo *Cryphonectria parasitica* no sería un buen ejemplo para avalar esta hipótesis de coevolución, de hecho, a la luz de este caso surge una nueva hipótesis de origen de los micovirus. El estudio comparado de secuencias de hypovirus con las de virus vegetales con genomas de ssRNA pertenecientes al género *Potyvirus*, revelan que existe una mayor relación de los hypovirus con los virus vegetales que con muchos de los micovirus asintomáticos conocidos. Surge así otra hipótesis que propone un ancestro común entre los hypovirus y los potyvirus de plantas, de manera que hongos patógenos o saprofíticos adquirieron virus con genomas de ssRNA de las plantas y posteriormente perdieron su cápsida proteica y evolucionaron a formas de dsRNA debido a diferentes presiones evolutivas (Pearson *et al.,* 2009). Ahora bien, la hipótesis inversa también podría contemplarse y los micovirus habrían pasado a tener por hospedador a la planta a la que el hongo parasitaba previamente, actuando el hongo como vector (Sabanadzovic *et al.,* 2009).

2.OBJETIVOS, METODOLOGÍA, RESULTADOS Y DISCUSIÓN

En esta sección de la memoria se expondrá el trabajo experimental y los resultados obtenidos durante la realización de esta Tesis Doctoral. La presentación del apartado se ha dividido en cinco capítulos diferentes, aunque todos ellos se incluyen dentro del objetivo global del trabajo, "*Estudio de micovirus asociados a hongos endofíticos y entomopatógenos"*.

Cada uno de los capítulos consta de manuscritos de artículos publicados o enviados a revistas internacionales, así como de manuscritos en preparación para su publicación.

Al comienzo de cada capítulo se ha añadido un pequeño resumen en castellano en el que se explican los antecedentes y objetivos del trabajo, así como un resumen de los principales resultados obtenidos.

Los capítulos incluidos en la presente memoria son:

CAPITULO I. Abundancia de micovirus en diferentes especies de hongos endofíticos de gramíneas.

Herrero, N., Sánchez Márquez, S., Zabalgogeazcoa, I. 2009. Mycoviruses are common among different species of endophytic fungi of grasses. *Archives of Virology*, 154, 327-330.

CAPITULO II. Patogenicidad contra garrapatas, termotolerancia e infección por virus en *Tolypocladium cylindrosporum*.

Herrero, N., Pérez-Sánchez, R., Oleaga, A., Zabalgogeazcoa, I. Tick pathogenicity, thermal tolerance and virus infection in *Tolypocladium cylindrosporum*. *Annals of Applied Biology*. Enviado.

CAPITULO III. Micovirus que infectan al hongo endofítico y entomopatógeno *Tolypocladium cylindrosporum*.

Herrero, N., Zabalgogeazcoa, I. Mycoviruses infecting the endophytic and entomopathogenic fungus *Tolypocladium cylindrosporum*. *Virus Research*. Enviado.

CAPITULO IV. Capacidad endofítica del hongo entomopatógeno *Tolypocladium cylindrosporum* en plantas de tomate y judía.

Herrero, N., Sánchez Márquez, S., Zabalgogeazcoa, I. Endophytic establishment of the entomopathogenic fungus *Tolypocladium cylindrosporum* in tomato and bean plants. Manuscrito en preparación.

CAPITULO V. Caracterización molecular y distribución de micovirus en el hongo entomopatógeno *Beauveria bassiana* en España y Portugal.

Herrero, N., Dueñas, E., Quesada-Moraga E., Zabalgogeazcoa, I. Molecular characterization and distribution of mycoviruses in the entomopathogenic fungus *Beauveria bassiana* in Spain and Portugal. Manuscrito en preparación.

Capítulo I. Abundancia de micovirus en diferentes especies de hongos endofíticos de gramíneas

Antecedentes

Como se ha descrito en la introducción general, los hongos endofíticos son un grupo de hongos que infectan partes aéreas o subterráneas de las plantas sin causar síntomas aparentes. Distintos estudios llevados a cabo durante los últimos veinte años han puesto de manifiesto que no existe una sola especie vegetal sin una micobiota endofítica asociada (Arnold, 2007; Hyde y Soytong 2008). Algunos de estos hongos endofíticos son capaces de mantener relaciones mutualistas con sus hospedadores. Así, pueden proteger a sus hospedadores de ciertos estreses bióticos como el herbivorismo o el ataque por patógenos vegetales, y también pueden conferir a sus hospedadores resistencia a ciertos estreses abióticos (Scharld *et al.,* 2004; Waller *et al.,* 2005; Zabalgogeazcoa, 2008). No obstante, para la mayor parte de las especies endofiticas conocidas se desconoce el efecto que producen en sus hospedadores.

Las asociaciones entre los virus de hongos y sus hospedadores son similares a las asociaciones descritas entre plantas y hongos endofíticos. De este modo, a diferencia de los virus animales o vegetales, los micovirus no producen una sintomatología obvia en sus hospedadores (Ghabrial y Suzuki, 2008). Ahora bien, existen algunos casos en que los micovirus producen un efecto claro en su hospedador, así, pueden producir hipovirulencia en algunos hongos fitopatógenos, enfermedad en champiñones, e incluso, pueden producir termotolerancia en el conjunto mutualístico formado por una gramínea, un hongo endofítico y su micovirus asociado (Deng *et al.,* 2005; Marquez *et al.,* 2007; Romaine y Goodin, 2002). Dado que hasta el momento no existía un estudio que demostrase cuán abundantes son los micovirus entre los hongos, en este capítulo se quiso estudiar si también existía un paralelismo entre las relaciones micovirus-hongo y las relaciones descritas entre endofitos y plantas en lo referido a su ubicuidad. Así, se estudió la abundancia de micovirus en el grupo de los hongos endofíticos no sistémicos de gramíneas.

Objetivos

1. Estudio de la incidencia y diversidad de micovirus en una colección del grupo de los hongos endofíticos no sistémicos de gramíneas.

Resultados

1. Incidencia de micovirus en hongos endofíticos no sistémicos de gramíneas

Para el estudio de incidencia de micovirus dentro del grupo de los hongos endofíticos no sistémicos de gramíneas se partió de 103 aislados pertenecientes a 53 especies diferentes escogidas al azar de entre una extensa colección de hongos endofíticos no sistémicos de gramíneas. De las 53 especies analizadas 12 resultaron estar infectadas por micovirus, obteniéndose una incidencia de 22.6% especies de hongos endofíticos infectadas.

2. Incidencia de micovirus en *Beauveria bassiana* y *Torrubiella confragosa*

Varios aislados fueron intencionadamente analizados de dos de las especies de hongos endofíticos en estudio que a su vez eran entomopatógenas, *Beauveria bassiana* y *Torrubiella confragosa.* Así, 66.7% de los aislados analizados de *B. bassiana* resultaron infectados. Por otra parte, 43 % de los aislados analizados de *T. confragosa* resultaron albergar micovirus. De este modo, el 22.6% de incidencia de micovirus obtenido para el total de especies endofíticas analizadas podría ser una infraestimación. Así, si se analizaran más aislados por especie hongo, la incidencia de micovirus entre el grupo de los endofitos no sistémicos de gramíneas podría aumentar.

3. Diversidad de micovirus entre los hongos endofíticos de gramíneas

La detección de micovirus se basó en la detección de elementos de dsRNA, dado que la mayor parte de los micovirus conocidos tienen genomas de dsRNA o de ssRNA pero con intermediarios de replicación de dsRNA. Así, se detectaron elementos de dsRNA con tamaños comprendidos entre 1.0 y 6.5 kbp. Además, algunos de los aislados infectados contenían un único elemento de dsRNA y otros albergaban hasta cuatro. De este modo,

aunque se necesitarían secuencias parciales de polimerasa o de proteína de cápsida para poder clasificar a los elementos de dsRNA detectados dentro de una familia de micovirus, las características del tamaño y número de estos dsRNAs permitió especular sobre su naturaleza. Según esto, los hongos endofíticos de gramíneas analizados podrían albergar a miembros de las familias, *Partitiviridae*, *Chrysoviridae* o *Totiviridae*. Además, algunas de las especies analizadas como *T. cylindrosporum* podrían albergar infecciones mixtas.

Brief Report

Mycoviruses are common among different species of endophytic fungi of grasses

Noemí Herrero, Salud Sánchez Márquez, Iñigo Zabalgogeazcoa

Instituto de Recursos Naturales y Agrobiología, CSIC. Apartado 257, 37071 Salamanca, Spain

Abstract

A survey of mycoviruses was made in a collection of 103 isolates belonging to 53 different species of endophytic fungi of grasses. Double-stranded RNA (dsRNA) elements were detected in isolates of 12 of the species analyzed. The banding characteristics and sizes of some of the dsRNA elements suggest that they might belong to previously described mycovirus families. The observed incidence (22.6%) indicates that the presence of mycoviruses could be common among species of this group of ubiquitous fungi.

Endophytes are a group of fungi which infect aerial or underground plant parts without causing any apparent symptoms in their hosts. Endophyte surveys carried out in the last twenty years indicate that most, if not all plant species, are hosts of these fungi. Furthermore, numerous endophytic species, sometimes more than a hundred, can be associated with a particular plant species (Arnold, 2007; Sánchez Márquez *et al.,* 2006; Stone *et al.,* 2004). Some fungal endophytes maintain mutualistic associations with their hosts. For example, seed transmitted *Epichloë* species systemically infect several species of grasses, producing alkaloids which make the plants more resistant to herbivores (Scharld *et al.,* 2004). Other endophytic species are known to improve disease resistance or abiotic stress tolerance in their hosts (Waller *et al.,* 2005; Zabalgogeazcoa, 2008). However, for most endophytic species the effects of the symbiotic association on their hosts are unknown.

The associations between fungal viruses and their hosts are similar to plant-endophyte associations. Unlike plant or animal viruses, which are commonly associated with disease, many of the known fungal viruses cause no obvious symptoms (Ghabrial, 1998; Ghabrial and Suzuki, 2008). Only a few mycoviruses are known to affect their hosts, causing hypovirulence or disease (Deng *et al.,* 2007; Romaine and Goodin, 2002), and some are beneficial. In fact, a mutualistic association between mycoviruses, endophytes, and their plant hosts, resulting in increased plant thermal tolerance, has been described recently (Marquez *et al.,* 2007). Mycoviruses have not received as much attention as animal or plant viruses, but numerous fungal viruses have been described since the first report of such a mycovirus was made by Hollings in 1962 (Hollings, 1962). Many of these viruses have double-stranded RNA (dsRNA) genomes, but viruses having single-stranded RNA (ssRNA), and double-stranded (dsDNA) genomes also exist (Ghabrial, 1998; Ghabrial and Suzuki, 2008). The purpose of the present investigation was to determine if mycoviruses were common in a collection of different species of endophytic fungi of grasses.

The fungal material analyzed consisted of 103 fungal isolates belonging to 53 species chosen randomly from a collection of endophytic fungi isolated from the grasses *Ammophila arenaria*, *Alopecurus arundinaceus*, *Brachypodium sylvaticum*, *Cynodon dactylon*, *Dactylis glomerata*, *Elymus farctus*, *Festuca rubra*, *Holcus lanatus*, and *Lolium perenne*. In endophyte surveys it is common for some species to be abundant, and for others to be represented by a single isolate (Arnold, 2007; Sánchez Márquez *et al.,* 2006; 2008). Because of this, in our collection some species were represented by a single isolate. The species from which we purposely examined more isolates were *Beauveria bassiana* and *Torrubiella confragos*a, two entomopathogenic fungi which have often been isolated as endophytes from grasses and other plant species (Bills, 1996; Sánchez Márquez *et al.,* 2006; 2008).

The screening for mycoviruses was based on the detection of dsRNA elements. This method allows the detection of genomes of dsRNA viruses, and of replicative forms of ssRNA viruses (Morris and Dodds, 1979). Fungal isolates were cultured for 15-20 days on cellophane disks layered on top of potato dextrose agar in Petri plates. Approximately one gram of fresh mycelium was ground with liquid nitrogen, and dsRNA was extracted by CF-11 cellulose chromatography (Morris and Dodds, 1979). The purified dsRNA was

treated with DNase I (Ambion TURBO DNA free), subjected to gel electrophoresis and the dsRNA elements visualized after staining with ethidium bromide.

DsRNA elements were detected in isolates belonging to 12 of the 53 endophytic species analyzed (Table 1; Fig. 1). The size of the dsRNA elements detected following electrophoresis of CF-11 cellulose extracts ranged from ca. 6.5 to 1.0 kbp (Table 1). Some infected isolates contained only one element, while others contained as many as four. The size of known mycovirus-associated dsRNA elements range from 13 kbp observed in the replicative forms of some members of the *Hypoviridae,* a family of single-stranded (+) RNA mycoviruses (Nuss *et al.,* 2005; Zhang and Nuss, 2008), to 1.4 kbp in the bipartite genomes of the *Partitiviridae,* a family whose members have dsRNA genomes (Ghabrial *et al.,* 2005a). Therefore, the size, as well as the number of most observed dsRNA fragments suggested that they may represent mycovirus genomes.

Fig. 1. Electrophoretic banding patterns of dsRNA elements isolated from *Tolypocladium cylindrosporum,* lane 1; *Fusarium culmorum,* 2; *Mastygobasidium intermedium,* 3; *Curvularia inaequalis,* 4; *Penicillium* sp. 5; *Gaeumannomyces graminis* 6; *Rhizoctonia bataticola,* 7. Two different dsRNA patterns were observed in different isolates of *Beauveriabassiana,* 8, 9, and *Torrubiella confragosa*, 10, 11. Lanes M contain λ-*Hin*d III size marker, numbers on left indicate kbp.

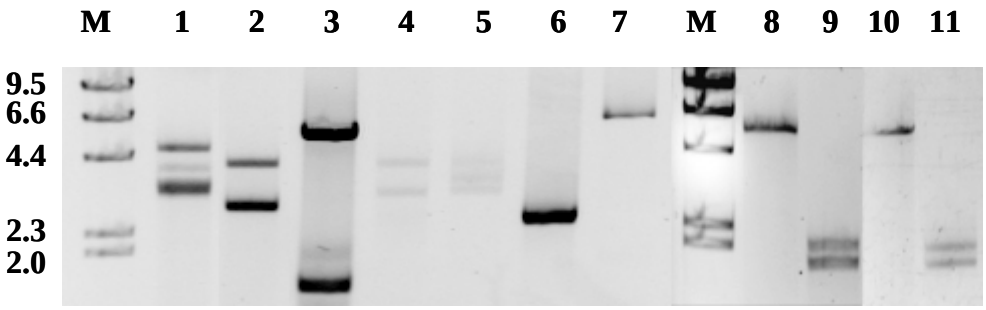


Table 1. Endophyte taxa where mycovirus-like dsRNA elements were detected. The host grass from where the fungus was isolated is shown in parentheses. Hosts from where endophytes were obtained were A: *Ammophila arenaria*, Al: *Alopecurus arundinaceus*, B: *Brachypodium sylvaticum*, C: *Cynodon dactylon*, D: *Dactylis glomerata*, E: *Elymus farctus*, F: *Festuca rubra*, H: *Holcus lanatus*, L: *Lolium perenne*, P: *Poa* sp. Size estimates were determined using agarose gel electrophoresis and dsDNA size markers.

Isolates infected / total analyzed	Host taxa	DsRNA elements observed[a]	
		Number	Size (kbp)
5/15	*Beauveria bassiana* (D, A, E, H)	1	6.0
5/15	*Beauveria bassiana* (D, A, E, H)	2	1.9 – 2.3
1/1	*Curvularia inaequalis* (A)	2	4.5 – 3.4
2/2	*Drechslera biseptata* (D)	2	1.0 – 1.5
1/3	*Fusarium culmorum* (D, F)	2	3 – 4.4
2/2	*Gaeumannomyces graminis* (H)	1	2.6
1/1	*Mastigobasidium intermedium* (H)	2	1.4 – 5.7
1/2	*Penicillium canescens* (D)	2	1.6 – 1.8
1/2	*Penicillium* sp. (P, C)	3	3.5 – 3.8 – 4.5
1/1	*Rhizoctonia bataticola* (A)	1	6.9
2/2	*Tolypocladium cylindrosporu*m (F, H)	4	3.4 – 3.7 – 4.2 – 5.1
2/7	*Torrubiella confragosa* (A, Al, B, D, E, L)	2	1.9 - 2.3
1/7	*Torrubiella confragosa* (A, Al, B, D, E, L)	1	6.0
1/1	*Valsa* sp. (D)	1	4.5

[a] No dsRNA was detected in any of the following endophytic species: *Acremonium strictum* (5 isolates analyzed), *Alternaria tenuissima* (1), *Ascochyta* sp. (1), *Aureobasidium pullulans* (1), *Botryosphaeria* sp. (1), *Chaetomium funicola* (1), *Chaetomium* sp. (4), *Cochliobolus sativus* (1), *Colletotrichum* sp. (1), *Coniothyrium cereale* (2), *Coprinellus radians* (1), *Cordyceps sinensis* (1), *Cryptococcus victoriae* (1), *Diaporthe viticola* (1), *Discula quercina* (1), *Drechslera dactylidis* (2), *Drechslera* sp. (3), *Fusarium oxysporum* (2), *Fusarium sporotrichoides* (1), *Gaeumannomyces cylindrosporus* (1), *Glomerella graminicola* (1), *Helgardia anguioides* (5), *Hypoxylon fuscum* (1), *Hypoxylon* sp. (1), *Lachnum* sp. (1), *Leptodontidium orchidicola* (3), *Leptosphaeria* sp. (1), *Mortierella* sp. (1), *Paecilomyces lilacinus* (1), *Penicillium janthinellum* (1), *Petriella guttulata* (1), *Phaeosphaeria nodorum* (1), *Phaeosphaeria* sp. (2), *Phoma herbarum* (1), *Plectosphaerella cucumerina* (1), *Podospora* sp. (3), *Pyrenochaeta* sp. (1), *Sporidiobolus* sp. (1), *Stemphylium solan*i (2), *Stilbella* sp. (2), *Volutella* sp. (1).

Although partial or complete sequences of the RNA-dependent RNA polymerase (RdRp) or the coat protein gene are required to assign individual elements to mycovirus families, some characteristics of the dsRNA elements we observed allow us to speculate on the identity of some of the mycoviruses present in infected isolates. For example, several endophytic species harboured dsRNA molecules of a similar size to those of a totivirus genome, but also to the replicative form of some still unclassified ssRNA mycoviruses (Howitt *et al.,* 2001; Xie *et al.,* 2006; Yokoy *et al.,* 1999). The family *Totiviridae* comprises viruses with dsRNA genomes consisting of a single linear molecule of 4.6 to 6.7 kbp (Wickner *et al.,* 2005). This could be the case for some *Beauveria bassiana* and

Torrubiella confragosa isolates carrying a 6.0 kbp dsRNA, *Mastigobasidium intermedium* (5.7 kbp), *Rhizoctonia bataticola* (6.5 kbp), *Tolypocladium cylindrosporum* (5.1 kbp), and *Valsa* sp. (4.5 kbp) (Table 1; Fig. 1). In *M. intermedium* and *T. cylindrosporum* the putative totivirus genomes, or ssRNA virus replicative forms of 5-6 kbp were accompanied by smaller dsRNA elements. This could indicate mixed infections by two or more viruses. Mixed infections by different mycoviruses, including totiviruses, have been reported in several fungal species (Ghabrial and Suzuki, 2008), including *Epichloë festucae*, a grass endophyte (Romo *et al.,* 2007). In the case of *T. cylindrosporum*, the minor dsRNA bands could correspond to the genome of a member of the family *Chrysoviridae*, whose genomes are composed of 4 segments of 2.4 to 3.6 kbp (Ghabrial *et al.,* 2005b). On the other hand, these small dsRNA fragments that accompany the ca. 5 kb dsRNAs, could also be replicative forms of ssRNA viruses (Yokoi *et al.,* 2003), satellite RNAs, or defective derivatives of replication (Yao *et al.,* 1995; Zhang and Nuss, 2008). An unidentified *Penicillium* species, *Curvularia inequalis*, and *Fusarium culmorum* also had dsRNA elements of size similar to that of the *Chrysoviridae*. Other species showed dsRNA patterns with characteristics of the family *Partitiviridae*, whose members have genomes composed of two dsRNA segments of 1.4-2.2 kbp (Ghabrial *et al.,* 2005a). This is the case for some of the *Beauveria bassiana* and *Torrubiella confragosa* isolates. The dsRNA patterns observed in these two clavicipitaceous entomopathogenic endophytes were very similar (Fig. 1). In endophytic *Beauveria bassiana* 10 out of 15 isolates that were analyzed contained dsRNA. Two different dsRNA banding patterns were observed in this species, and half of the infected isolates harboured one dsRNA element ca. 6.0 kbp in size, while the other half had two dsRNAs ca. 1.9 and 2.3 kbp in size (Fig. 1). The incidence of virus infection in the endophytic isolates of this species was 67 %, greater than the 17 % observed in isolates obtained from soil in other studies (Melzer and Bidochka, 1998). In *Torrubiella confragosa* 3 out of 7 isolates were infected. The banding patterns in this species were very similar to those observed in *Beauveria bassiana*, with one type of infection comprising a single dsRNA element ca. 6.0 kbp in size, and the other type comprising two bands ca.1.9 and 2.3 kbp in size.

The occurrence of dsRNA elements and viruses has been previously reported in some of the fungal genera analyzed in this study including *Beauveria bassiana*, in which dsRNA elements, ca. 2 kbp in size, similar to the ones we describe here have been observed (Dalzoto *et al,* 2006; Melzer and Bidochka, 1998). Also a mycovirus with a

similar dsRNA banding pattern to the one found in *Fusarium culmorum* here has been reported for *F. graminearum* (Chu *et al.,* 2004) and some of the numerous viruses described in *Gaeumannomyces graminis* have genomic dsRNA elements similar to the ones we show here (Jamil *et al.,* 1984). Also, a 6.4 kbp dsRNA associated with hypovirulence occurs in some isolates of *Rhizoctonia solani* (Tavantzis *et al.,* 2002). In contrast, *Curvularia* thermal tolerance virus has a genome consisting of two segments ca. 2.1 and 1.9 kbp in size (Marquez *et al.,* 2007), different from the larger dsRNAs we observed in *Curvularia inaequalis* (Fig. 1, Table 1), and while dsRNAs associated with hypovirulence have been described in *Valsa* sp. (Hammar *et al.,* 1989), to our knowledge the presence of virus-like dsRNAs had not been reported previously in any species of *Mastigobasidium*, *Tolypocladium*, or *Torrubiella.* The sizes and banding patterns of the dsRNAs found in the various endophytic fungi suggests that mycoviruses from several known families are represented in our cohort, but until some molecular characterisation is available they can not be assigned to any specific family.

Twenty-three percent of the 53 species analyzed contained virus-like dsRNA elements. Furthermore, this incidence of mycoviral infection among species could be an underestimation because several species that we analyzed were represented by a single isolate. Due to the apparently asymptomatic nature of their infection, endophytic fungi were almost unknown until twenty years ago, but now they are considered ubiquitous organisms (Ghabrial and Suzuki, 2008). A similar situation exists with fungal viruses, and since the first mycovirus was observed (Hollings, 1962), many more have been described (Ghabrial and Suzuki, 2008). In conclusion, the results of this survey suggest that mycoviral infections are relatively common among species of endophytic fungi of grasses.

Acknowledgments

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CAPITULO II. Patogenicidad contra garrapatas, termotolerancia e infección por virus en *Tolypocladium cylindrosporum*

Antecedentes

Tolypocladium cylindrosporum resultó ser una de las especies infectadas en el estudio de incidencia de micovirus en hongos endofíticos no sistémicos de gramíneas. Este hongo además de colonizar plantas como endofíto, es capaz de colonizar otros hábitats, así, es un hongo típicamente aislado de suelo, pero también es un conocido patógeno de especies de mosquitos que son vectores de patógenos que causan importantes enfermedades como la malaria, el dengue o la fiebre amarilla (Scholte *et al.*, 2004). *T. cylindrosporum* también es capaz de infectar otras especies de insectos e incluso arácnidos como las especies de garrapatas *Ornithodoros erraticus* y *Ornithodoros moubata* (Lam *et al.*, 1988; Zabalgogeazcoa *et al.*, 2008), las cuales son importantes vectores de distintos patógenos animales y humanos (Oleaga-Pérez *et al.*, 1990; Vial, 2009). Por otra parte, el género *Tolypocladium*, *T. cylindrosporum* incluido, es también conocido por la producción de ciclosporina. La ciclosporina es una sustancia muy preciada en medicina ya que se emplea para evitar rechazos en los trasplantes de órganos (Aarnio y Agathos, 1989). Por estas características comentadas sobre *T. cylindrosporum* en esta tesis se ha prestado especial atención al estudio de diversos aspectos de la biología de este hongo, así como al estudio de sus micovirus asociados.

En la actualidad, los principales medios para el control de garrapatas se basan en el uso de acaricidas químicos. Estos compuestos son efectivos pero tienen ciertas desventajas como su elevado coste, el desarrollo de resistencias, o la contaminación ambiental y de alimentos que pueden producir. Así, para evitar este tipo de problemas se ha evaluado el uso de hongos entomopatógenos como bioacaricidas. Varias cepas de hongos entomopatógenos com *B. bassiana* y *M. anisopliae* se han evaluado con este fin (Kaaya y Hassan, 2000), y en este capítulo se pretendió hacer lo mismo con *T. cylindrosporum*. Adicionalmente, se han descrito micovirus en otras especies de hongos entomopatógenos, como *B. bassiana, Metarhizium* spp., o *Paecilomyces* spp. (Dalzoto *et al.*, 2006; Inglis y Valadares-Inglis, 1997; Martins *et al.*, 1999; Melzer y Bidochka, 1998), ahora bien, aunque dos de estos trabajos proponen a un micovirus como el agente causal de hipervirulencia en

M. anisopliae o de hipovirulencia en *B. bassiana* (Dalzoto *et al.*, 2006; Melzer y Bidochka, 1998), hasta el momento, no se han descrito efectos claros producidos por micovirus en hongos entomopatógenos, al igual que ocurre con la mayoría de hongos infectados por virus.

Objetivos

1. Estudio de la capacidad acaricida de varias cepas de *T. cylindrosporum* contra las especies de garrapatas *O. erraticus* y *O. moubata.*

2. Estudio de la respuesta a altas temperaturas de *T. cylindrosporum*.

3. Estudio de la incidencia de micovirus en *T. cylindrosporum*, e influencia de los micovirus en la patogenicidad y en la respuesta a las altas temperaturas de *T. cylindrosporum.*

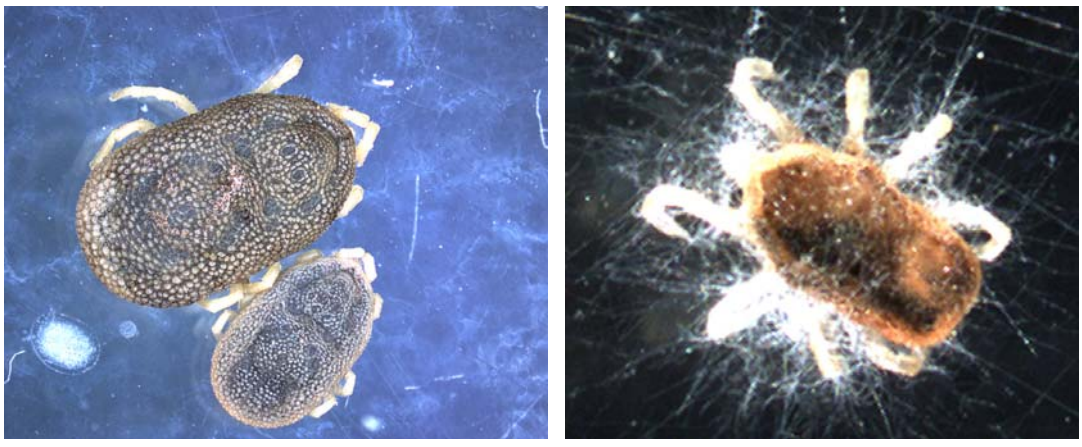
Resultados

1. Capacidad acaricida de *T. cylindrosporum* contra las especies de garrapatas *O. erraticus* y *O. moubata*

Todos los estadíos evolutivos de ambas especies de garrapatas, *O. erraticus* y *O. moubata* fueron susceptibles a las cinco cepas de *T. cylindrosporum* usadas en el ensayo (Fig. 1). No obstante, *O. erraticus* fue la especie más susceptible a *T. cylindroporum*, alcanzándose porcentajes de mortalidad para todos los estadíos evolutivos de esta especie muy cercanos al 70% con cuatro de las cepas evaluadas.

Fig. 1. A. *O. erraticus* macho y hembra sanos. **B.** *O. erraticus* infectado por *T. cylindrosporum.*

A **B**



2. Efecto de la temperatura en el crecimiento de *T. cylindrosporum*

El crecimiento radial de las once cepas de *T. cylindrosporum* en estudio fue significativamente mayor a 22 ºC que a 30 ºC. En promedio, los diámetros de los aislados a 30 ºC fueron 59% más pequeños que los observados a 22 ºC. Por otro lado, se encontraron diferencias significativas entre los crecimientos de los distintos aislados a las distintas temperaturas. También se observó la existencia de una correlación significativa entre el porcentaje de reducción de crecimiento de las cepas a 30 ºC y el diámetro de crecimiento de éstas a 30 ºC.

3. Incidencia de micovirus en *T. cylindrosporum*

Cinco de las 11 cepas de *T. cylindrosporum* analizadas resultaron albergar micovirus. El tamaño de los dsRNAs detectados en las distintas cepas varió de 1.2 a 5.1 kbp. Además, todas las cepas infectadas contenían al menos dos moléculas diferentes de dsRNA.

4. Influencia de los micovirus en la patogenicidad y la respuesta a las altas temperaturas de *T. cylindrosporum*

La presencia de micovirus no afectó significativamente a los diámetros medios de los 11 aislados de *T. cylindrosporum* en estudio a 22 ºC y a 30 ºC. Lo mismo ocurrió con la patogenicidad de *T. cylindrosporum* hacia *O. erraticus* y *O. moubata*, que no fue afectada significativamente contra ninguna de las dos especies de garrapatas por la carga viral.

Tick pathogenicity, thermal tolerance and virus infection in *Tolypocladium cylindrosporum*

Noemí Herrero, Ricardo Pérez-Sánchez, Ana Oleaga, Iñigo Zabalgogeazcoa

Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain

Abstract

Tolypocladium cylindrosporum is a fungus which has been isolated from soil, from asymptomatic plants as an endophyte, and has been shown to be pathogenic to several species of arthropods. The objective of the present work was to study a collection of *T. cylindrosporum* strains in order to evaluate the characteristics of this fungus as a bioacaricide. The pathogenicity of five different strains of *T. cylindrosporum* was tested against two tick species, *Ornithodoros erraticus* and *Ornithodoros moubata*. Both tick species were susceptible to all the fungal strains. Mortality was greater for *O. erraticus*, and differed among the five developmental stages of the ticks which were tested, and among the fungal treatments. Mean mortality rates were close to 60% for *O. erraticus*, similar to those reported for other entomopathogenic fungi used for this purpose. The responses of eleven different strains of the fungus to 22 ºC and 30 ºC were also studied. Significant differences in temperature tolerance occurred among the strains, and growth inhibition was observed at 30 ºC. Several mycoviruses were found infecting five of the eleven strains. However, no clear relationship was found between the presence of viruses and fungal growth or pathogenicity.

Keywords: Biological control, entomopathogens, mycovirus, endophyte, Argasidae, *Ornithodoros*

Introduction

The fungal genus *Tolypocladium* is well known because of its biotechnological importance due to the production of cyclosporine (Aarnio and Agathos, 1989). *Tolypocladium cylindrosporum* produces cyclosporine A, a cyclic peptide that exhibits a strong and selective immunosuppressive activity, and became a crucial agent for the development of transplant surgery across histocompatibility barriers (Aarnio and Agathos, 1989). This compound is also used for the treatment of autoimmune and parasitic diseases, as well as in cancer chemotherapy (Reed and Thomas, 2008).

T. cylindrosporum was first reported as a soil-borne species, isolated from a variety of soils and turfs (Gams, 1971), and later it was found causing epizootics in mosquito populations of *Aedes sierrensis*, *Aedes australis*, and *Culex tarsalis* (Soares, 1982; Weiser and Pillai, 1981). Although *T. cylindrosporum* was originally thought to have a life cycle spent in soil and insects, this and other entomopathogenic species have been isolated as fungal endophytes capable of asymptomatically infecting plant tissues (Quesada-Moraga *et al*., 2006a; Sánchez Márquez *et al.*, 2010; Vega *et al.*, 2008).

In addition to cyclosporine A, *T. cylindrosporum* is known to produce high amounts of tolypin and efrapeptins, substances which are toxic to insects (Bandani *et al.*, 2000; Weiser and Matha, 1988). After these discoveries were made, this entomopathogenic fungus has been considered as a potential control agent for several mosquito genera, including *Anopheles* and *Aedes*, that are vectors of pathogens causing important human diseases such as malaria, dengue, and yellow fever (Scholte *et al.*, 2004). *T. cylindrosporum* has a wide host range, in addition to dipterans is pathogenic to insects of other orders such as *Lepidoptera* and *Ephemeroptera*, as well as to crustaceans like *Daphnia carinata* and *Tigriopus* spp. (Lam *et al.*, 1988). Recently a strain of this fungus has been reported as a pathogen of the argasid ticks *Ornithodoros erraticus* and *Ornithodoros moubata,* which have great medical and veterinary importance as vectors of African swine fever virus and several species of human relapsing fever borreliae (Zabalgogeazcoa *et al.*, 2008). *O. erraticus* is distributed in the Mediterranean basin and, in southern Europe it lives in close association with swine on free range pig farms, hidden in holes and fissures inside and around pig-pens (Manzano-Román *et al*., 2007; Oleaga-Pérez *et al*., 1990). *O. moubata* is distributed throughout South and East Africa and Madagascar, where it colonizes wild and domestic habitats and feeds on warthogs, domestic swine, and

humans (Vial, 2009). To date, the primary means of tick control are based on the use of chemical acaricides. These compounds are very effective but have disadvantages such as higher costs, development of resistance, or environmental and food contamination. In order to overcome these problems researchers have evaluated the use of entomopathogenic fungi as bioacaricides. Many strains of *Beauveria bassiana* and *Metarhizium anisopliae* have been tested for this purpose (Kaaya and Hassan, 2000).

Viruses have been identified in many fungal species, including entomopathogens like *T. cylindrosporum, Beauveria bassiana, Metarhizium*, and *Paecilomyces* spp. (Herrero *et al.*, 2009; Inglis and Valadares-Inglis, 1997; Martins *et al.*, 1999; Melzer and Bidochka, 1998). A few mycoviruses are known to affect their hosts, causing hypovirulence in plant and insect pathogens, or growth distortion in mushrooms (Ghabrial and Suzuki, 2009; Melzer and Bidochka, 1998). However, unlike animal or plant viruses, most known fungal viruses rarely cause obvious symptoms in their hosts. For example, virus-infected strains of the entomopathogen *Metarhizium anisopliae* do not show a distinct culture phenotype, reduced conidiospore production *in vitro*, or virulence against insects or ticks (Frazzon *et al.*, 2000; Melzer and Bidochka, 1998). Nevertheless, the fact that fungal viruses are common among fungi, that their vertical transmission to spores is often very efficient, and that their infections are very persistent, suggests that under some conditions some viruses could be beneficial to their hosts (Ghabrial and Suzuki, 2008; Herrero *et al*., 2009; Romo *et al*., 2007). For instance, a mutualistic virus which improves the thermal tolerance of a plant-endophyte association has been recently reported (Marquez *et al.*, 2007).

The main purpose of this research was to characterize a collection of *T. cylindrosporum* strains in order to evaluate the potential of this fungus as a bioacaricide. We studied how temperatures higher than optimal affected the growth of eleven *T. cylindrosporum* strains, and tested the pathogenicity of five different isolates against two species of *Ornithodoros* ticks. In addition, we analyzed the presence of mycoviruses in these strains, and studied if the presence of viruses had an effect on fungal pathogenicity or growth at higher temperatures.

Materials and methods

Fungal strains and taxonomy

The *T. cylindrosporum* isolates used in this study (Table 1) were obtained from the collections of the Centraalbureau voor Schimmelcultures (CBS), Colección Española de Cultivos Tipo (CECT), and the Merck, Sharp and Dohme collection (MSD). Strains 3398 and 11 were isolated as fungal endophytes from asymptomatic plants of the grasses *Holcus lanatus* and *Festuca rubra* in natural grasslands of western Spain (Sánchez Márquez *et al.*, 2010). Strain 3398M is a subculture obtained from strain 3398 which spontaneously lost one of the six dsRNA elements infecting the original 3398 strain. This characteristic was maintained in the strain after being stored in the laboratory and subcultured several times.

All isolates were identified according to morphological characteristics (Bissett, 1983), as well as with the nucleotide sequence of their ITS1-5.8SrRNA-ITS2 region. These sequences were obtained using the method described by Sánchez Márquez *et al.* (2007). Sequences of other taxa were retrieved from the EMBL nucleotide database (http://www.ebi.ac.uk/embl/). For sequence-based identification all sequences were aligned using the program ClustalX (Thompson *et al.*, 1997). A species dendrogram was constructed with MEGA 3.1 software using the neighbor-joining method and a Tajima-Nei model to calculate distances (Kumar *et al.*, 2004).

Table 1. *Tolypocladium cylindrosporum* strains used in the study. Strain T2 is the type strain of the species (Gams, 1971).

Strain	Source	Country of origin	Original substrate
3398	IRNASA	Spain	*Holcus lanatus*[a]
3398M[b]	IRNASA	Spain	*Holcus lanatus*
11	IRNASA	Spain	*Festuca rubra*[a]
T1	CBS 719.70	Czech Republic	Soil
T2	CBS 718.70	UK	Peat soil
T4	CBS 276.82	New Zealand	Mosquito
T5	CBS 612.80	USA	Mosquito
T6	CBS 550.75	Argentina	Soil
T7	CECT 20414	Ireland	Mosquito
T8	MSD	Unknown	Unknown
T9	MSD	Unknown	Unknown

[a]*Holcus* and *Festuca* are two genera of grasses. [b] Strain 3398M was derived from a subculture of strain 3398 which spontaneously lost a 5.1 kbp dsRNA element.

Growth response to temperature

To study isolate responses to a temperature higher than optimal, the eleven strains were cultured in potato dextrose agar (PDA) Petri plates at two different temperatures, 22 ºC and 30 ºC. Preliminary tests carried with isolates 11 and 3398M showed that in the conditions used for this study, the isolates did not grow at 32 ºC. Bissett (1983) reported that 22 ºC is the optimal temperature for growth on PDA for all the species of the genus.

A small block of mycelium, measuring about 3 x 3 mm was placed in the center of a 9 cm PDA plate which was sealed with Parafilm. Six plates of each strain were placed in two incubators set at 22 and 30 ºC. The position of the 66 culture plates incubated together at each temperature was randomized. After 21 days of incubation at each temperature, the diameter of the fungal cultures was measured (Zabalgogeazcoa *et al.*, 1998). At each temperature, the statistical significance of the differences on the diameters of the fungal strains was analyzed using a one way ANOVA followed by an LSD procedure. Values of $p < 0.05$ were considered significant. To check if there was a relationship between the percentage of growth inhibition observed in each isolate at the higher temperature, and its diameter at 22 ºC or 30 ºC, simple linear correlation (Pearson correlation) was used. The statistical analyses were done using the Statistica 5.0 (StatSoft, USA) software package.

Pathogenicity on ticks

The *O. erraticus* and *O. moubata* ticks came from two colonies maintained in our laboratory. The colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain, and the colony of *O. moubata* from specimens obtained from the Institute for Animal Health, Pirbright, Surrey, UK. These ticks are fed regularly on rabbits, and kept at 28 ºC and 80 % relative humidity (RH).

Five strains of *T. cylindrosporum* were chosen for this assay, 11 and 3398M were obtained as grass endophytes, T4 was isolated from a mosquito host, and T1 and T6 were isolated from soil (Table 1). To obtain conidial suspensions, each strain was grown on PDA Petri plates at room temperature (22–25 ºC). Conidia from 3-week old cultures were released from the mycelium with a glass rod, after adding 5 ml of sterile water containing 0.01% Tween 80 to each plate. The conidial suspensions from the plates were collected and centrifuged at 2000 x g for 5 min. The pellets were resuspended in sterile water and the concentration of conidia was estimated with a Bürker chamber (Zabalgogeazcoa *et al.*, 2008). To prepare these suspensions from *T. cylindrosporum*, on average we obtained 1.5 x

10^7 spores from each gram of fresh mycelium. A 20 day culture on a PDA Petri plate contained about 1.5 g of mycelium.

Five developmental stages from both *Ornithodoros* species were treated with the five strains of *T. cylindrosporum*. Namely, males, females, nymphs-4, nymphs-3 (20 individuals of each stage), and nymphs-2 (50 individuals). Three replicate groups were made from each tick species and developmental stage for testing each fungal strain. Each group of three replicates was inoculated in parallel at the same time. All the tick specimens used were newly moulted and unfed at the start of the pathogenicity experiments. For inoculation each treatment group was placed in a vial containing 2 ml of the corresponding conidial suspension (10^8 conidia/ml in water containing 0.01% Tween 80). After 5 minutes, the excess suspension was removed, and the ticks were incubated for 60 days at 28 ºC and 80% RH. Also in parallel, three replicate groups of each tick species and developmental stage were treated with a 0.01% Tween 80 aqueous solution without conidia, and used as a negative control. Mortality was recorded for every group at 3, 7, 14, 28 and 60 days post-inoculation (d.p.i.), and the percentage of cumulative mortality was calculated.

For the statistical analyses, the mortality data was transformed as the arcsin of the square root of the proportion of dead ticks. The normality of the transformed data of each observation period (d.p.i.) was tested using a Kolmogorov-Smirnov test. Differences in mortality between tick species, developmental stages, and fungal treatments, were analysed at each observation period (3, 7, 14, 28, and 60 d.p.i.) using three way ANOVA. The control treatment was not included in the statistical analyses. Comparisons of the total mortality caused by each fungal strain in each tick species at each observation period were made using LSD values obtained from one way ANOVAs for each observation period with fungal strain as main effect, and pooled values for all developmental stages of the ticks. Values of $p < 0.01$ were considered significant.

Analysis of the presence of double-stranded RNA

The presence of double stranded RNA (dsRNA) molecules of sizes ranging from 1 to 12 kbp was used as a method to diagnose virus infection in isolates. This type of nucleic acid can represent the genome of dsRNA mycoviruses, as well as replicative intermediates of viruses with single-stranded RNA genomes (Morris and Dodds, 1979). However, not all

RNA viruses can be detected by dsRNA isolation (de Blas *et al*., 1996), and DNA viruses, recently discovered in fungi (Yu *et al*., 2010), would not be detected with this technique.

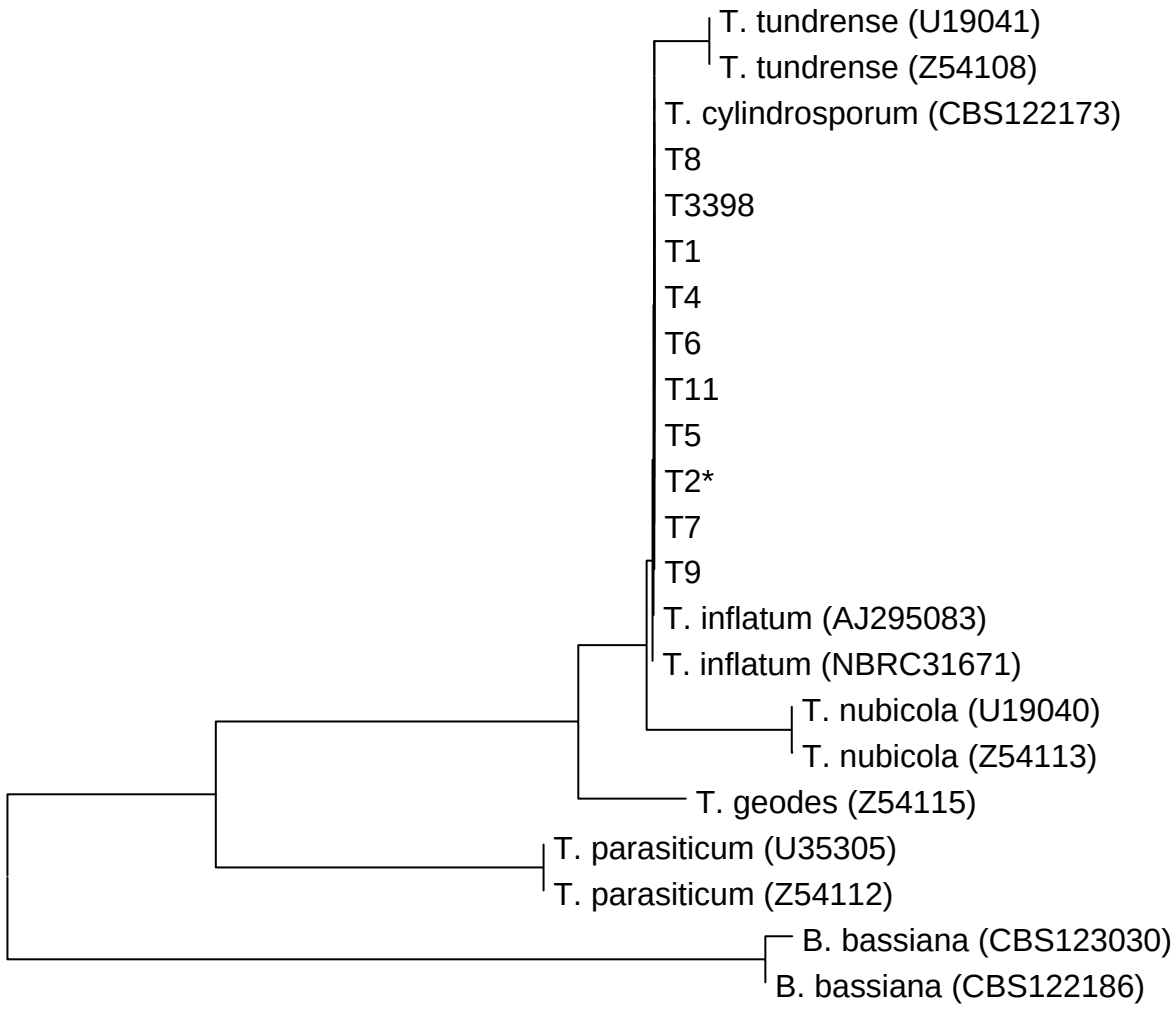
To determine if dsRNA might be present in the *T. cylindrosporum* strains, fungal isolates were cultured for three weeks over cellophane disks layered on top of PDA in Petri plates. Approximately 1.5 grams of fresh mycelium were harvested, ground with liquid nitrogen, and dsRNA was extracted by CF-11 cellulose chromatography (Morris and Dodds, 1979). The purified dsRNA samples were treated with DNase I (Ambion TURBO DNA-free), subjected to gel electrophoresis, and visualized after staining with ethidium bromide.

Results

Isolate identification

All eleven strains listed in Table 1 had the characteristic cylindrical conidia of *T. cylindrosporum* (Bissett, 1983). In addition, their ITS1-5.8SrRNA-ITS2 nucleotide sequences, including that of the type strain of *T. cylindrosporum* (strain T2, CBS718.70), were identical. In a dendrogram based on nucleotide sequences, all *T. cylindrosporum* strains grouped together in a clade separated from those of other species of the genus included in this analysis (Fig. 1). The *T. inflatum* sequence differed only by four single nucleotide gaps from the *T. cylindrosporum* sequence. Therefore, morphological and molecular characters indicated that all our strains belonged to the same species: *T. cylindrosporum*.

Fig. 1. Dendrogram built using sequences from the ITS1-5.8SrRNA-ITS2 region of *T. cylindrosporum* isolates included in the study. Other reference sequences were obtained from the EMBL nucleotide database; numbers between parentheses indicate their accession numbers. The dendrogram was constructed using the neighbor-joining method and a Tajima-Nei model to calculate distances. The type strain of *T. cylindrosporum* (T2) is indicated by an asterisk.



Effect of temperature on growth of fungal strains

The radial growth of all strains was greater at 22 ºC than at 30 ºC (Table 2). The higher temperature was not favorable for fungal growth, one strain did not grow, and on average, culture diameters at 30 ºC were about 59% smaller than those observed at 22 ºC (Table 2). ANOVA indicated significant differences in growth among strains at 22 ºC ($F_{10,55} = 340.97$; $P<0.01$), as well as at 30 ºC ($F_{10,55} = 98.92$; $P<0.01$). At 22 ºC isolate diameters ranged from 47.25 to 13.92 mm. At 30 ºC isolate T1 did not grow at all, in

concordance with its slow growth at the optimum temperature. Isolate T2 was also quite sensitive to the higher temperature, and the remaining cultures showed growth rates double or triple that the one of T2 at 30 ºC. At this temperature, strains 3398, T5, T8 and T9 showed growth rates significantly greater ($p < 0.05$) than those of the remaining strains (Table 2).

Table 2. Average diameter of cultures and percentage of growth inhibition observed at 30 ºC in eleven strains of *Tolypocladium cylindrosporum* after 21 days of growth at 22 ºC and 30 ºC.

Strain	**Diameter 22ºC (mm)**	**Diameter 30ºC (mm)**	**Diameter reduction at 30ºC (%)**
3398	36.5	24.8	32.1
T5	46.5	23	50.5
T8	45.6	20.8	54.4
T6	39.1	17.4	55.5
T9	44.9	19.5	56.6
11	33.3	14.1	57.7
3398M	37.1	14.9	59.8
T4	43.3	14.1	67.4
T7	47.3	12.6	73.4
T2	39.6	8.7	78.0
T1	13.9	0	100
[a]LSD; d.f.	1.45; 55	1.99; 55	

[a]Least significant difference and degrees of freedom; $p<0.05$

When the percentage of growth inhibition among strains at 30 ºC was estimated, a wide range of values, ranging from 100 to 32%, was observed (Table 2). There was a strong correlation between the percentage of inhibition and the average diameter of each strain at 30 ºC ($r = -0.96$, $p < 0.01$), and a weak correlation at 22 ºC, ($r = -0.54$, not significant). Therefore, the diameter of a strain at 30 ºC could be considered as a good indicator of its heat tolerance.

Pathogenicity of fungi to O. erraticus and O. moubata

The five strains of *T. cylindrosporum* were pathogenic to both tick species (Figs. 2-4). In both *Ornithodoros* species, dead ticks appeared swollen and with red coloration in their cuticle and legs. About seven days after death, fungal mycelium showing morphological characteristics of *T. cylindrosporum* were observed on the surface of dead ticks.

Fig. 2. Cumulative mortality (%) in males, females, nymphs 4 (N4), nymphs 3 (N3), and nymphs 2 (N2) of *O. erraticus* at 3, 7, 14, 28, and 60 days post inoculation with *T. cylindrosporum* strains T1, T4, T6, 3398M and 11. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia.

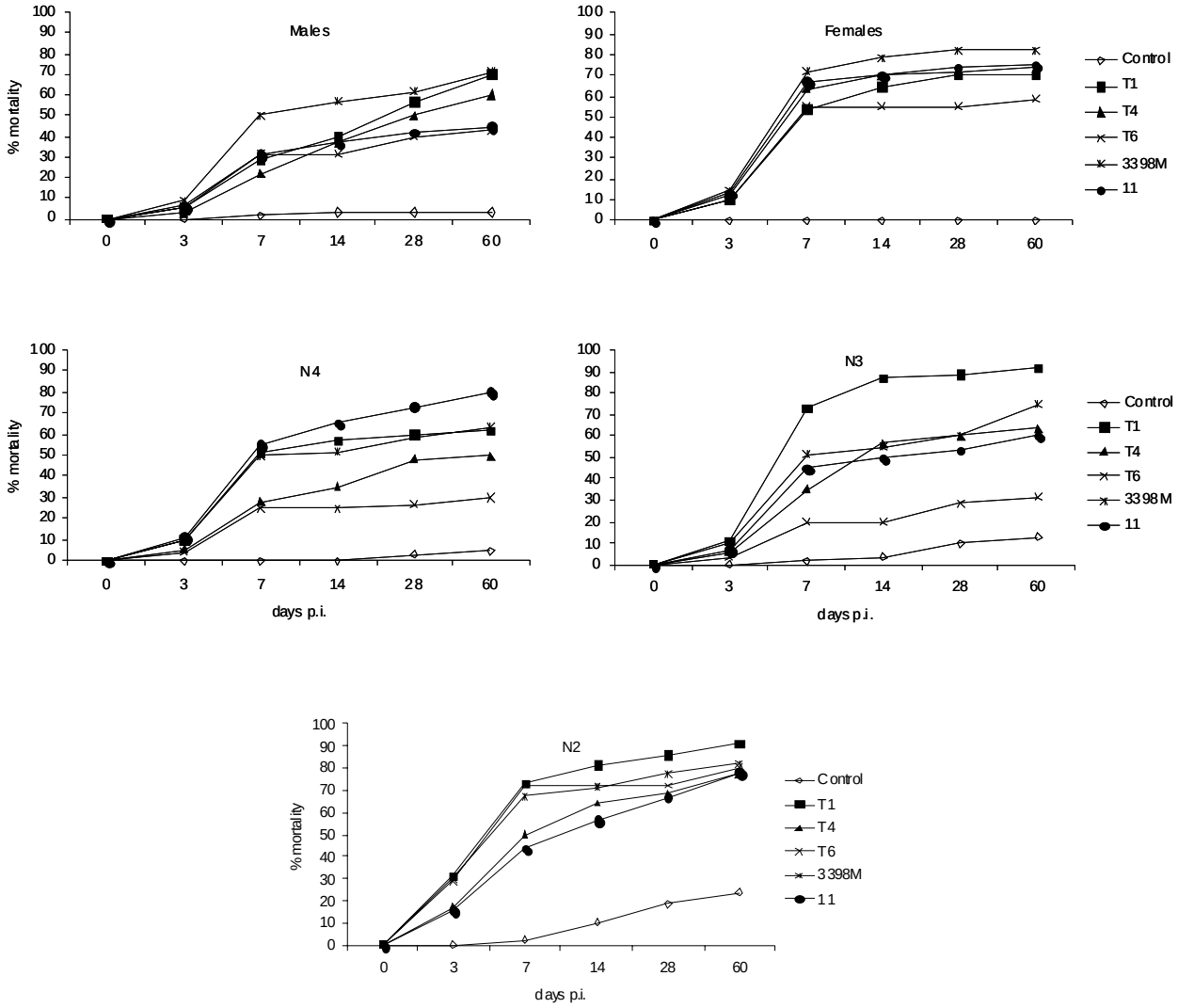


Fig. 3. Cumulative mortality (%) in males, females, nymphs 4 (N4), nymphs 3 (N3), and nymphs 2 (N2) of *O. moubata* at 3, 7, 14, 28 and 60 days postinoculation with *T. cylindrosporum* strains T1, T4, T6, 3398M, and 11. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia.

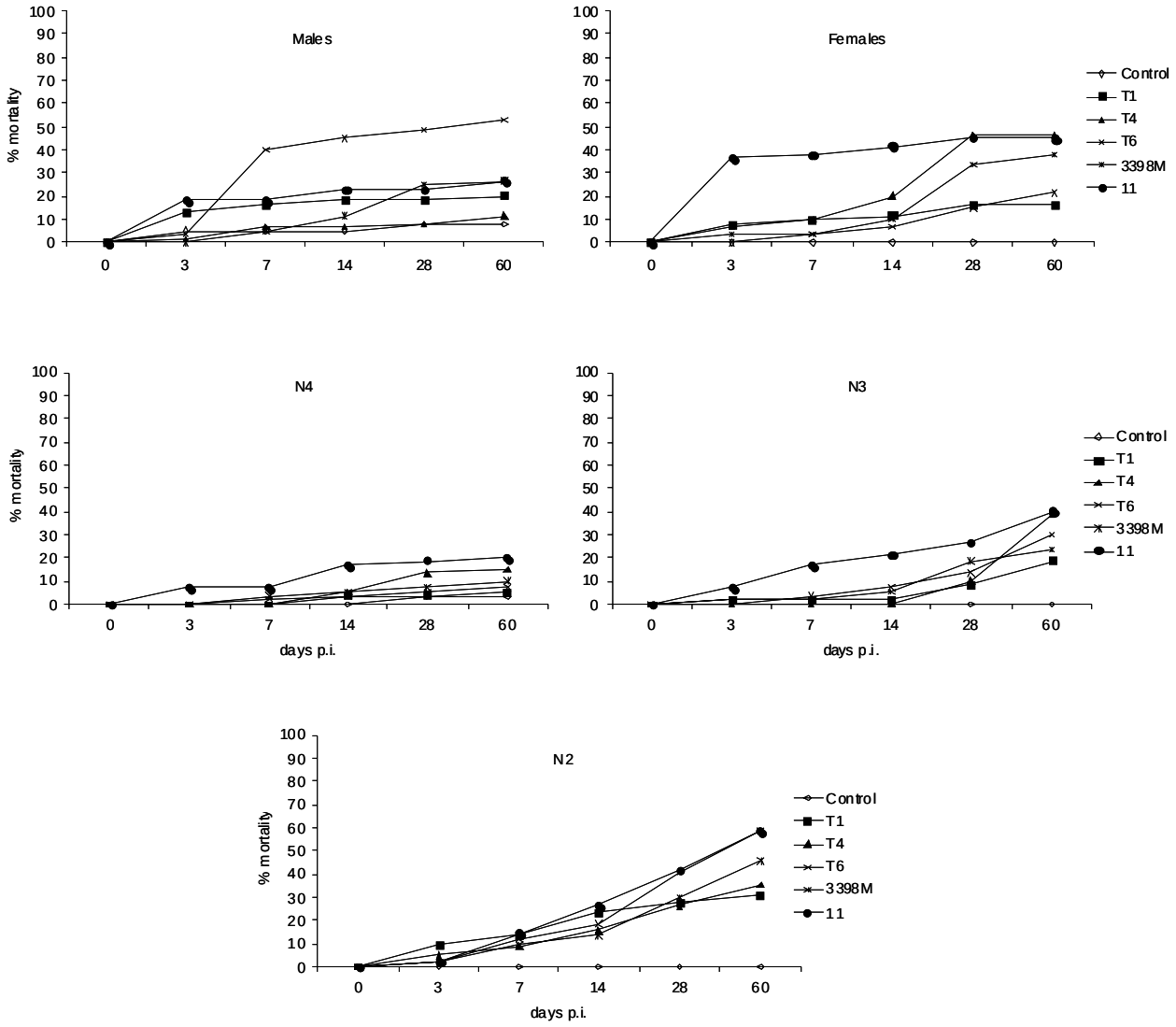
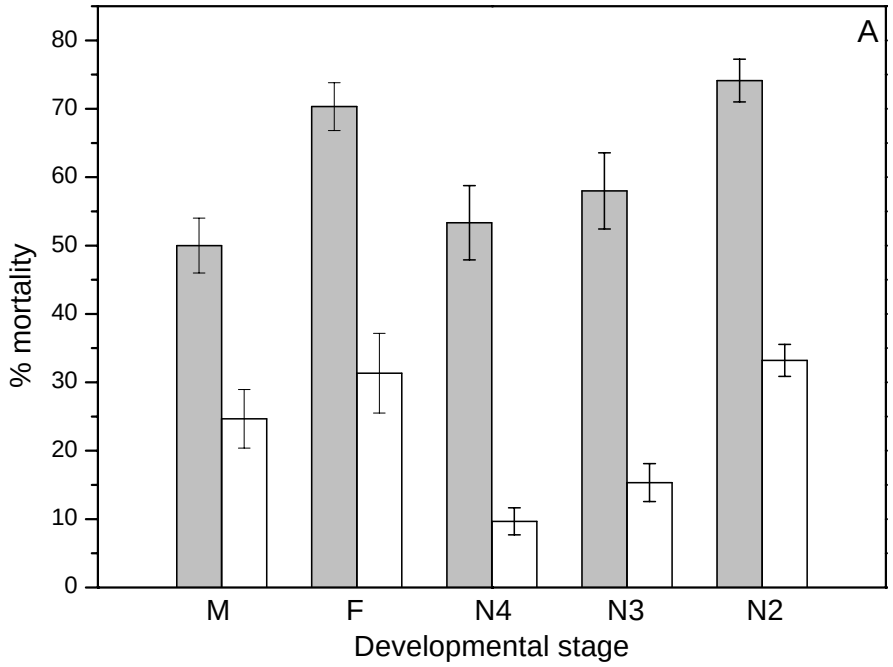
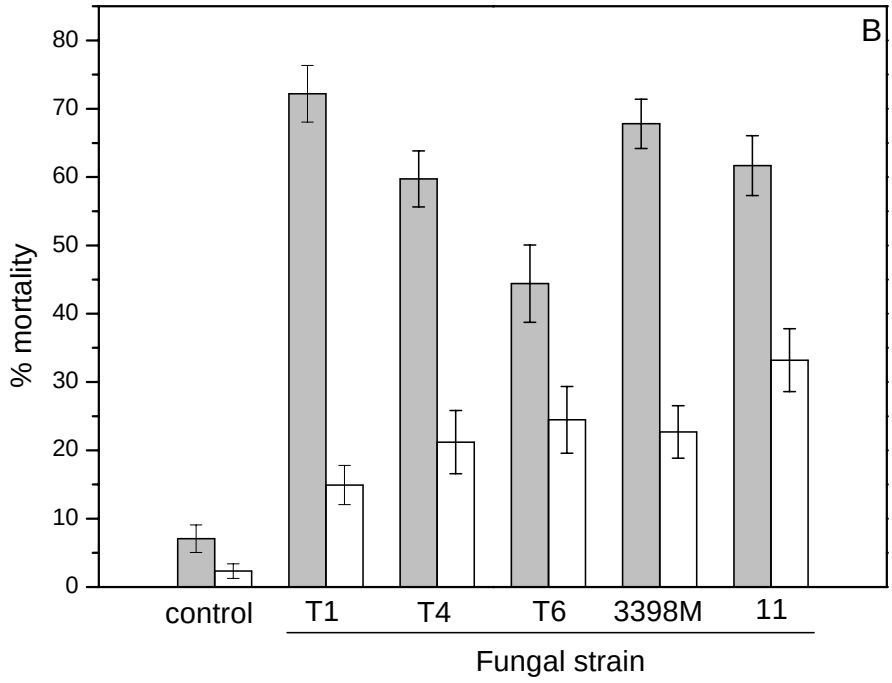


Fig. 4. **A.** Mortality (mean ± standard error) observed on each developmental stage of *O. erraticus* (grey bars) and *O. moubata* (white) at 28 days post inoculation. **B.** Mortality (mean ± standard error) caused by each fungal strain in each tick species in the same time period. M, males; F, females; N4, nymphs-4; N3, nymphs-3; N2, nymphs-2.





Mortality was significantly greater for *O. erraticus* than for *O. moubata* (Table 3; Figs. 2-4). All developmental stages of *O. erraticus* were susceptible to all the strains of *T. cylindrosporum* tested (Figs. 2, 4A). In most cases, the mortality of ticks began as early as 3 d.p.i., and after that increased rapidly, slowing down after 7 d.p.i. Little or no additional mortality took place between 28 and 60 d.p.i. The exception to this rule were the males treated with fungal strains T1 and T4, whose mortalities increased constantly throughout the whole period observed. At 60 d.p.i. all fungal strains except T6 induced mean mortality rates between 65% and 77%. In *O. moubata* mortality started at 3 d.p.i. and increased slowly until 60 d.p.i. (Fig. 3). Mortality rates at 60 d.p.i. varied considerably among developmental stages and fungal strains, hardly surpassing 50%.

Table 3. F values produced by the analyses of variance of mortality data at each of five periods post inoculation.

Effect	df	Days post inoculation				
		3	**7**	**14**	**28**	**60**
Tick species (T)	1	550.79**	469.91**	460.25**	297.21**	249.33**
Stage (S)	4	11.50**	13.70**	15.83**	17.58**	20.14**
Fungus (F)	4	8.71**	5.60**	6.51**	3.38*	2.70*
TxS	4	4.28**	10.86**	9.03**	2.95*	1.48 ^{ns}
TxF	4	5.58**	6.65**	11.01**	9.67**	10.48**
SxF	16	2.19**	2.67**	2.60**	2.41**	2.28**
TxSxF	16	1.16 ^{ns}	1.78*	2.50**	1.41 ^{ns}	1.39 ^{ns}

** p<0.01; * p<0.05; ns: not significant

Significant differences in susceptibility to the fungi were observed among developmental stages of both tick species (Figs. 2, 3, 4A). Although mortality was greater for *O. erraticus* than for *O. moubata*, the same trend in mortality according to the developmental stage was observed in both tick species (Fig. 4A); females and nymphs-2 had the greatest mortality rates. As a result, a significant interaction between tick species and developmental stage was indicated by the ANOVA (Table 3). Similarly, the mortality caused by each fungal strain was greater for *O. erraticus* than for *O. moubata* (Fig. 4B), resulting in a significant interaction between tick species and fungal strain. In addition, there was an interaction between developmental stage and fungal strains (Table 3). In *O. erraticus*, at all observation dates there were significant differences among some fungal treatments in the mortality of nymphs-3, and at four dates among nymphs-4 (Fig. 2). In this

tick species there were significant differences in mortality among some developmental stages at four dates for the treatments with strains T1 and T6, and at 3 dates for strain T4. In *O. moubata*, at all observation dates there were significant differences among some fungal treatments in the mortality of males, at three dates among nymphs-4, and at one date among nymphs-3 and nymphs-2 (Fig. 3). There were significant differences among some developmental stages at four dates for treatment T6, at 3 dates for T4, and at one date for T1.

Considering the total mortality caused for all developmental stages, some fungal strains appeared more virulent than others. In *O. erraticus*, strain T6 was significantly less pathogenic than the others at several dates. In contrast, in *O. moubata* strain T1 was significantly less virulent than the other strains at several d.p.i. (Table 4).

Table 4. Transformed values (arcsin[proportion of dead ticks]^{½}) of the mean mortality across all developmental stages observed on each tick species after each observation period. The significance of the differences between the transformed means can be determined using the LSD ($p<0.01$).

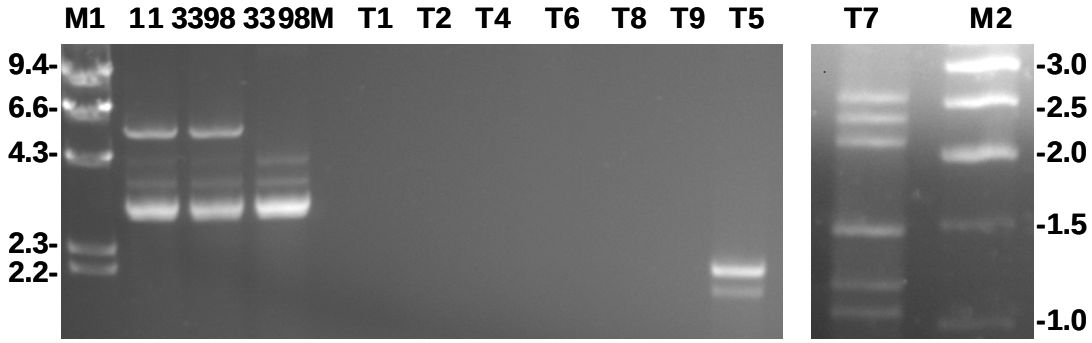
Days post inoculation	**Fungal strain**					
	T1	**T4**	**T6**	**3398M**	**11**	**[a]LSD; d.f.**
O. erraticus						
3	0.78	0.62	0.62	0.84	0.72	0.19; 70
7	0.85	0.67	0.68	0.87	0.77	0.20; 70
14	0.98	0.81	0.68	0.92	0.85	0.21; 70
28	1.05	0.89	0.73	0.98	0.91	0.20; 70
60	1.11	0.94	0.79	1.06	0.99	0.21; 70
O. moubata						
3	0.19	0.09	0.04	0.07	0.31	0.17; 70
7	0.22	0.174	0.266	0.174	0.406	0.20; 70
14	0.28	0.266	0.358	0.276	0.52	0.19; 70
28	0.35	0.45	0.49	0.47	0.58	0.20; 70
60	0.41	0.55	0.60	0.55	0.65	0.21; 70

[a] Least significant difference; degrees of freedom.

Presence of viruses in T. cylindrosporum strains

Mycoviral dsRNA elements were detected in five of the eleven strains analyzed (Fig. 5). The electrophoretic patterns observed revealed the existence of molecules of 11 different sizes, ranging from 1.2 to 5.1 kbp (Table 5). The size of these dsRNA molecules are within the range of sizes observed in mycovirus genomes (Ghabrial and Suzuki, 2008). All infected isolates contained at least two different dsRNA molecules. Isolates 3398 and 11, both obtained as endophytes from different grass species (Table 1), had an identical dsRNA electrophoretic pattern.

Fig. 5. Electrophoretic banding patterns of dsRNA elements present in eleven *Tolypocladium cylindrosporum* isolates. Lanes M1 and M2 contain size markers and numbers on left and right indicate size in kbp.



Strain 3398M had the same pattern as 3398 and 11, except for a 5.1 kbp band, which was not present. Strain 3398M was derived from a culture of strain 3398 which lost the 5.1 kbp dsRNA element. Therefore, this might indicate that strains 11 and 3398 are infected by more than one virus, and one of them was lost in isolate 3398M. Totiviruses have genomes consisting of a single dsRNA molecule of 4.5 to 7.0 kbp (Wickner *et al.*, 2005); the 5.1 kbp dsRNA band lost from isolate 3398 could have been a totivirus genome. The sets of dsRNA molecules detected in T5 and T7 are completely different from those detected in the strains obtained from grass endophytes. Both strains were obtained from mosquito hosts (Table 1).

Table 5. *T. cylindrosporum* isolates where mycovirus-like dsRNA elements where detected. Size estimates were determined using agarose gel electrophoresis and dsDNA size markers.

Strain	dsRNA elements observed	
	Number	**Size (kbp)**
3398M	3	3.1 - 3.2 - 3.4 - 3.7 - 4.2
3398	4	3.1 - 3.2 - 3.4 - 3.7 - 4.2 - 5.1
11	4	3.1 - 3.2 - 3.4 - 3.7 - 4.2- 5.1
T5	2	1.8 - 2.2
T7	6	1.2 - 1.3 - 1.5 - 2.2 - 2.3 - 2.6

The presence of mycoviruses did not seem to affect the average diameter of cultures of the isolates at 22 and 30 ºC. No statistical significance was found when a Student's t-test ($p < 0.05$) was used to test the difference between the mean diameters of virus-infected and virus-free isolates. However, we observed a significant difference in the growth of strains 3398 and 3398M. These strains are isogenic, but 3398M is not infected by the 5.1 kbp dsRNA. At 22 ºC the two strains did not differ significantly in their growth, but at 30 ºC, the diameter of strain 3398 was significantly greater ($p < 0.05$) than that of 3398M.

At each developmental stage, the mean tick mortality caused by all virus-infected strains was compared to that caused by virus-free strains using a Student's t-test. No statistically significant differences between means were observed for any developmental stage. These results suggest that mycoviruses do not seem to affect the pathogenic processes of these fungi against *O. erraticus* and *O. moubata.*

Discussion

Fungi have been the main agents used in the early work in biological control developed between the 19th and 20th centuries, this might be because they are the most visible insect pathogens, and most are readily culturable (Lord, 2005). Nevertheless, research on fungi for control of ticks is rather new, because chemical acaricides are generally used for this purpose. However, biological control is becoming an attractive approach for tick management (Fernandes and Bittencourt, 2008; Kaaya and Hassan, 2000).

T. cylindrosporum is an interesting fungus that has not received as much attention as other entomopathogens traditionally used in biological control. Several characteristics of this fungus make it a very good candidate as a potential biological controller. The species has a very good sporulation capability; the strains we analyzed produced about $1.5x10^7$ spores per gram of fresh mycelium. Its spores are very resistant to temperatures under 0 ºC; in our laboratory, a suspension of spores kept at -20 ºC for one year germinated efficiently. This characteristic might be useful for long term storage of spores. In addition, spores from *T. cylindrosporum* are capable to persist in the soil for long periods of time (Bissett, 1983). All the *T. cylindrosporum* isolates we included in the study had the same ITS1-5.8SrRNA-ITS2 nucleotide sequence as the type strain of the fungus, and could be distinguished from other related species (Fig. 1). Therefore this molecular character seems useful for the identification of individuals of the species.

Another good characteristic of this species for its use in biological control is that several strains showed a relatively low growth inhibition at the higher temperature tested, and these strains might perform better than others in warm environments. To match the thermal tolerance of a prospective fungal strain to the climatic conditions expected at the target environment where it is going to be applied is desirable (Quesada-Moraga *et al.,* 2006b). We found that there is a good correlation between the percentage of growth inhibition observed at 30 ºC, and the average diameter of a strain at 30 ºC. Therefore, a screening for heat tolerant strains could be done in a collection by means of selecting those strains having the largest diameters at 30 ºC.

The results of pathogenicity tests showed that *T. cylindrosporum* has a good potential for the biological control of ticks. The five different strains examined (3398M, 11, T1, T4 and T6) were pathogenic to both argasid species, *O. erraticus* and *O. moubata*, but their virulence varied notably among tick species and developmental stages (Fig. 4). With the exception of T6, about one month after inoculation all strains induced mean mortality rates higher than 60% against *O. erraticus.* These rates are similar to those reported in the literature for *B. bassiana* and *M. anisopliae* in ixodid ticks, which range from 20% to 100% (Fernandes *et al.*, 2003; Samish *et al.*, 2004). On the other hand, all the strains of *T. cylindrosporum* were less effective against *O. moubata*. Strain T1 caused the lowest mean mortality rate (18%) in this species. This is curious because strain T1 caused the highest mortality rate in *O. erraticus*, showed the smallest diameter at 22 ºC, and was

not viable at 30 ºC. This fact suggests that there might be no relation between growth rate and pathogenicity of *T. cylindrosporum,* at least in the experimental conditions used.

The presence of dsRNA molecules was observed in 5 of the 11 isolates analysed. These dsRNAs were detected in strains of different geographical origin (Ireland, Spain, and USA), obtained from different substrates (grass leaves and dead mosquitoes). All dsRNA elements observed (Table 5) presented sizes similar to those of mycovirus genomes, which range from 13 kbp in the replicative forms of some members of the Hypoviridae family (Nuss *et al.,* 2005) to 1.4 kbp in the bipartite genomes of the Partitiviridae family (Ghabrial *et al.,* 2005a). This incidence of mycoviruses is comparable to those detected in other entomopathogenic fungi such as *Beauveria bassiana, Torrubiella confragosa,* and *Metarhizium anisopliae* (Herrero *et al.,* 2009; Melzer and Bidochka, 1998), and indicates that as in other insect pathogens, the presence of viruses seems to be common in *T. cylindrosporum.*

A clear relationship between the presence of viruses and pathogenicity was not found. This result is in concordance with those obtained in similar experiments performed with strains of *M. anisopliae,* the presence of dsRNA did not affect the virulence of *M. anisopliae* strains against ticks or insects (Frazzon *et al.,* 2000; Giménez-Pecci *et al.,* 2002). In the same way, the presence of viruses did not seem to affect the radial growth of fungal strains grown at 22 or 30 ºC. However, it is interesting that strains 3398 and 3398M, which differ in the presence of a 5.1 kbp dsRNA molecule, but presumably not in the fungal genotype, showed different behaviors at 30 ºC (Table 3). It is possible that the presence of the 5.1 kbp dsRNA, which could represent a totivirus genome, might confer some advantages at the higher temperature to the fungus. However, strain 11 has a dsRNA pattern similar to 3398 (Fig. 5), and its behavior at 30 ºC was like that of strain 3398M.

In conclusion, *T. cylindrosporum* has good characteristics to be considered as a tick control agent. It is known the fungus sporulates abundantly, and its spores are persistent in storage. The present work shows that the species can be easily separated molecularly from other members of the *Tolypocladium* genus, and strains are pathogenic against all stages of *O. erraticus*, and to a less extent *O. moubata*. Although mycoviruses are present, these did not appear to affect pathogenicity.

Given that some strains are functional at higher than optimal temperatures (30 ºC) and the host range is known to be wide, this fungus may have the potential to be applied topically as a mycoinsecticide to eliminate at the same time ticks and other arthropod pests

in livestock (Polar *et al.*, 2008). However, additional studies are necessary in order to better understand the effects of dsRNA on conidiogenesis, persistence, production of secondary metabolites, and other important traits that could interfere with the performance of virus infected strains as biological control agents.

Acknowledgments

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CAPITULO III. Micovirus que infectan al hongo endofítico y entomopatógeno *Tolypocladium cylindrosporum*

Antecedentes

Como se ha comentado en el capítulo anterior *T. cylindrosporum* es un hongo que entraña un elevado interés debido a su versatilidad en cuanto a los hábitats que es capaz de colonizar, a su potencial como biocontrolador, y como productor de sustancias de interés médico. Debido a estas características y al hecho de que las cepas endofíticas de *T. cylindrosporum* estudiadas en los dos capítulos anteriores albergan micovirus, en este capítulo se trató de identificar y secuenciar a los virus que infectan a estas cepas endofíticas. Aunque se han encontrado micovirus infectando otros hongos entomopatógenos como *B. bassiana*, *M. anisopliae* o *Paecilomyces* spp. (Bidochka *et al.,* 2000; Dalzoto *et al.,* 2006; Inglis y Valadares-Inglis, 1997), ninguno de ellos ha sido identificado hasta la fecha. Así, el conocimiento del genoma de estos virus puede ayudar a entender mejor las relaciones tritróficas virus-hongo-artrópodo o virus-hongo-planta. No obstante, el estudio de este tipo de interacciones entre los micovirus y sus hospedadores se ha visto desfavorecido por el hecho de que la mayoría de estas infecciones son muy persistentes y difíciles de eliminar (Martins *et al.,* 1999; Romo *et al.,* 2007), además, hasta el momento no existen medios eficaces de curación para estas infecciones o medios efectivos de inoculación artificial de micovirus, que ayudarían a este tipo de investigaciones (Ghabrial y Suzuki, 2009). Así, en este capítulo se probó el uso del antiviral ribavirín en la curación de los micovirus que infectan a *T. cylindrosporum*.

Objetivos

1. Estudio de la transmisión a las esporas asexuales de los micovirus que infectan a *T. cylindrosporum.*

2. Secuenciación e identificación de micovirus asociados a *T. cylindrosporum*.

3. Curación de infecciones virales en *T. cylindrosporum* con la aplicación del antiviral ribavirín.

4. Estudio de la influencia micovirus en la respuesta a las altas temperaturas de *T. cylindrosporum.*

Resultados

1. Transmisión a las esporas asexuales de los micovirus que infectan a *T. cylindrosporum*

El hongo endofítico y entomopatógeno *T. cylindrosporum* resultó albergar infecciones mixtas por micovirus, como así se dedujo de este estudio de trasmisión a los conidios de los diferentes dsRNAs albergados por las cepas 11 y 3398M. La cepa 11 alberga a TcV1, TcV2 y TcV3, mientras que la cepa 3398M está infectada solamente por TcV2 y TcV3. TcV1 mostró la tasa más alta de transmisión en la cepa 11, 81.4%, mientras que TcV3 sólo fue transmitido a un 4.7% de los conidios. En la cepa 3398, TcV2 fue transmitido al 90% de las esporas asexuales, pero TcV3 no se transmitió a los conidios.

2. Secuenciación e identificación de micovirus asociados a *T. cylindrosporum*

TcV1 fue totalmente secuenciado. Así, su genoma está formado por una molécula de dsRNA de 5196 bp que contiene dos fases de lectura abierta (ORFs) que codifican una RNA polimerasa dependiente de RNA (RdRp) y una proteína de cápsida (CP) (Apéndice 1). Un análisis filogenético basado en las secuencias de aminoácidos de RdRps y CPs de otros micovirus de la familia *Totiviridae*, demostró que TcV1 constituye un nuevo miembro del género *Victorivirus*. De hecho, TcV1 constituye el primer micovirus que infecta a un hongo entomopatógeno que ha sido totalmente secuenciado e identificado.

También se obtuvo la secuencia completa de uno de los dsRNAs que componen el genoma cuatripartito de TcV2. Este dsRNA tiene un tamaño de 3486 bp y contiene un ORF que codifica para una RdRp (Apéndice 2). Adicionalmente, un estudio filogenético demostró que es muy probable que TcV2 constituya un nuevo miembro de la familia *Chrysoviridae*. Ahora bien, no se obtuvo ningún clon para TcV3. No obstante, se comprobó que los tres micovirus estaban encapsidados, de hecho, se purificaron partículas virales de TcV1.

3. Curación de micovirus con el antiviral ribavirín

Dos concentraciones diferentes del antiviral ribavirín (80 y 100 μM) se probaron para curar a las dos cepas endofíticas de *T. cylindrosporum* de sus infecciones por micovirus. TcV1 resultó muy sensible al fármaco. Todos los aislados monospóricos derivados de la cepa infectada tratada con las dos concentraciones diferentes de ribavirín fueron curados de la infección por TcV1. En cambio, TcV3 resultó bastante resistente a las concentraciones de ribavirín del ensayo. El efecto que el fármaco tuvo sobre TcV2 fue difícil de evaluar debido a la baja tasa de transmisión a las esporas asexuales que mostró este virus. De acuerdo con los resultados obtenidos, el antiviral probado en este ensayo tiene efectividades diferentes según las especies de micovirus tratadas.

4. Influencia de los micovirus en la respuesta a las altas temperaturas de *T. cylindrosporum*

No se observaron efectos claros atribuibles a un determinado virus en la respuesta de *T. cylindrosporum* a las altas temperaturas.

Mycoviruses infecting the endophytic and entomopathogenic fungus *Tolypocladium cylindrosporum*

Noemí Herrero and Iñigo Zabalgogeazcoa

Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC). Cordel de Merinas 40-52, 37008 Salamanca, Spain.

Abstract

Mixed mycoviral infections in the endophytic and entomopathogenic fungus *Tolypocladium cylindrosporum* were deduced from a study of the transmission to conidia of several double-stranded RNA (dsRNA) elements harboured by two fungal strains. Rates of transmission for each dsRNA were different, because isolates harbouring different combinations of the original set of five or six dsRNAs were obtained. A 5196 bp dsRNA element was sequenced and represents the genome of *Tolypocladium cylindrosporum* virus 1 (TcV1), a new member of the genus *Victorivirus* in the *Totiviridae* family. This virus was transmitted to 81.4% of the conidia produced by a strain with a multiple virus infection. Four dsRNAs of 3.1-3.7 kbp were transmitted only to 4.7% of the monosporic isolates produced by an infected isolate, and to none in the case of the other infected isolate. These four dsRNAs did not show segregation during transmission, one of them was sequenced and encoded a RdRp, suggesting that the four dsRNAs might represent the whole genome of a multipartite chrysovirus. A third virus with a genome of approximately 4.2 kbp was transmitted to 79.1% and 90% of the monosporic isolates produced by the two infected strains. None of the viruses, or their combinations, clearly affected the radial growth of infected fungal isolates incubated at 22 ºC or 29 ºC. Ribavirin was used to cure *T. cylindrosporum* from viruses, and TcV1 was sensitive to this drug. All monosporic cultures derived from an infected strain treated with two different concentrations of the drug (80-100 μM) were free of this virus.

Keywords: mycovirus, dsRNA, ribavirin, transmission, Victorivirus

Introduction

The ascomycete *Tolypocladium cylindrosporum* (Fam. *Ophiocordycipitaceae*) was first reported as a soil-borne species (Gams, 1971). Later it was found to be pathogenic to several species of insects, including mosquito genera like *Anopheles* and *Aedes,* which are vectors of pathogens causing human diseases such as malaria, dengue, and yellow fever (Lam *et al.,* 1988; Scholte *et al.,* 2004; Weiser and Pillai, 1981). The fungus is also pathogenic to crustaceans, and arachnids like the ticks *Ornithodoros erraticus* and *Ornithodoros moubata,* which are vectors of African swine fever virus, and several species of human relapsing fever borreliae (Zabalgogeazcoa *et al.,* 2008). In addition, this fungus has been reported as an endophyte, asymptomatically infecting the leaves of some grasses (Sánchez Márquez *et al.,* 2010). Other entomopathogenic fungi like *Beauveria bassiana*, *Lecanicillium lecanii,* or *Metarhizium anisopliae* have also been reported as endophytes and soil inhabitants, and they have been proposed and used as biological control agents for invertebrate plant pests (Meyling and Eilenberg, 2007; Vega *et al.,* 2008). The genus *Tolypocladium* is also known because some of its species, *T. cylindrosporum* included, produce cyclosporine A, a cyclic peptide that exhibits a strong and selective immunosuppressive activity and is used for organ transplants (Fritsche *et al.,* 2004).

Several families of fungal viruses have double-stranded RNA (dsRNA) genomes encapsidated in isometric particles, but unencapsidated viruses with single-stranded RNA (ssRNA) genomes, or single-stranded DNA (ssDNA) mycoviruses also exist (Ghabrial and Suzuki, 2008; Yu *et al.,* 2010). No extracellular route of infection is known for these mycoviruses, they seem to be transmitted intracellularly during cell division, sporogenesis, or cell fusion between compatible strains. Unlike plant or animal viruses, most known fungal viruses seem to be avirulent, and do not produce obvious symptoms in their hosts. Only a few mycoviruses are associated to phenotypic effects, causing hypovirulence in fungal pathogens, disease in mushrooms, or even being beneficial, conferring thermal tolerance to the grass host of a virus-infected endophyte (Hillman and Suzuki, 2004; Marquez *et al.,* 2007; Romaine and Goodin, 2002).

The presence of dsRNA molecules of viral origin has been reported in endophytic strains of *T. cylindrosporum,* as well as in other species of entomopathogenic fungi such as *B. bassiana, M. anisopliae* and *Paecilomyces* spp. (Bidochka *et al.,* 2000; Dalzoto *et al.,*

2006; Herrero *et al.,* 2009; Inglis and Valadares-Inglis, 1997). However, in none of these cases were these viruses classified.

Mycoviral infections can be very persistent, and difficult to eliminate from their hosts (Martins *et al.,* 1999; Romo *et al.,* 2007). Many attempts to cure fungi from viruses using different treatments have been reported, e.g., cycloheximide treatments, single conidium subculture, hyphal tip transfer, incubation at low or high temperatures (Carroll and Wickner, 1995; Marquez *et al.,* 2007; Romo *et al.,* 2007; Souza Azevedo *et al.,* 2000), but they were not always successful. These difficulties to cure fungi from viral infections, and the lack of simple methods for the artificial inoculation of mycoviruses, have greatly hampered progress in exploring mycovirus-host interactions (Ghabrial and Suzuki, 2009).

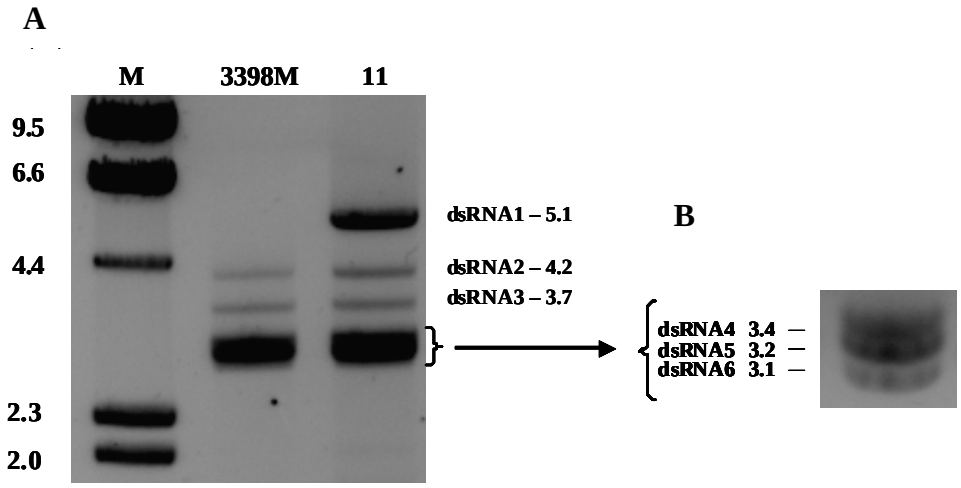
In this work we report the existence of mixed virus infections in two strains of *T. cylindrosporum*. The evidence for mixed infections came from the segregation of the dsRNA elements during their transmission to conidiospores, and from the sequencing of two dsRNA elements, one corresponding to a complete virus genome, and another to a component of a multipartite virus genome. In addition, the antiviral drug ribavirin was used to cure virus infected strains.

Materials and Methods

Fungal strains

Two strains of *T. cylindrosporum* were used in this study. Strain 11 was isolated as an endophyte from asymptomatic leaves of the grass *Festuca rubra* in natural grasslands of western Spain. This strain harboured six dsRNA elements with sizes approximately 5.1, 4.2, 3.7, 3.4, 3.2, and 3.1 kbp (Herrero *et al.,* 2009). These dsRNA elements will be referred according to their size, dsRNA1 being the largest, and dsRNA6 the smallest (Fig. 1). Strain 3398M was derived from an endophytic strain isolated from the grass *Holcus lanatus* (Sánchez Márquez *et al.,* 2010). The original strain (3398) harboured six dsRNA elements like those found in strain 11, but the dsRNA1 molecule was lost in a subculture of this strain named 3398M. This characteristic dsRNA composition remained stable in this strain.

Fig. 1. A. Electrophoretic banding patterns of the dsRNA elements present in strains 11, and 3398M of *T. cylindrosporum.* Lane M contains λ-*Hin*dIII size marker; numbers on the left and right indicate kbp. **B.** Enlarged picture of the bands inside the frame following extended electrophoresis.



Transmission of dsRNA elements to asexual spores

To determine the rate of transmission of the different dsRNA elements to asexual spores (conidia), monosporic isolates obtained from strains 11 and 3398M were analyzed to determine if all original dsRNA elements from the parental strain were transmitted. A set of 43 monosporic isolates derived from strain 11, and another of 40 monosporic isolates derived from strain 3398M were analyzed for the presence of dsRNA as explained in next section. To obtain monosporic isolates, both parental strains, which were monosporic isolates as well, were grown in potato dextrose broth (PDB) under shaking (110 rpm) for 12 days at 24 ºC. After this period the cultures were filtered trough sterile gauze, and the conidial suspensions in the filtrate were centrifuged for 5 min at 800 x g. Pellets were resuspended in 200 µl of water and different dilutions of these suspensions were made and added to water agar plates. Single germinated conidia were collected under the microscope with a needle, and plated into potato dextrose agar (PDA) plates to obtain each monosporic isolate.

This study of transmission of dsRNA elements to conidia had three main objectives: to determine if equal rates of transmission were obtained for all the dsRNAs carried by strains 3398M and 11, as reported in the literature for other fungi harbouring

mixed virus infections (Chu *et al.,* 2004; Romo *et al.,* 2007; Tuomivirta and Hantula, 2005); to be used as a control for the ribavirin curing experiment, since this study was carried parallel to this curing experiment; and to obtain cured isogenic isolates.

DsRNA purification and cDNA synthesis

Strain 11 was used to synthesize DNA complementary to some dsRNA elements harboured by this isolate. The strain was cultured for three weeks over cellophane disks layered on top of PDA plates. After this period the mycelium was harvested, and dsRNA was extracted by CF-11 cellulose chromatography (Morris and Dodds, 1979). To eliminate contaminating DNA, the purified dsRNA was treated with 5 units of DNase1 for 30 min at 37 ºC, and extracted with 1 vol. of phenol:chloroform (1:1). After that, contaminating ssRNA was removed by treatment with 1 unit of S1 nuclease at 37 ºC for 15 min. To check its quality, an aliquot of the purified dsRNA was electrophoresed in a 1% agarose gel, and visualized after staining with ethidium bromide (Fig. 1).

Approximately 2 µg of dsRNA dissolved in water were used for cDNA synthesis. DsRNA was denatured by heating at 95 ºC for 10 min in the presence of an excess (10 µg) of a degenerate oligonucleotide named Totioligo (5'-TTGAA(A/G)TC(A/G)TC(A/G)TA(A/G)TC(G/C)A(A/G)CA-3'). The design of this oligonucleotide was based on the alignment of the RNA-dependent RNA polymerase (RdRp) sequences of seven members of the *Totiviridae* family (Table 1). The nucleotide sequences of motif IV of the RdRps were the most conserved among these viruses, and the Totioligo sequence was based on this motif. The heat-denatured mixture was cooled in liquid nitrogen, and cDNA was synthesized using the Universal RiboClone cDNA Synthesis System (Promega). The cDNA synthesis products were adenylated at the 3' end by incubation in a solution containing Taq. polymerase and 10 mM dATP, and cloned in vector pGEM-T (Promega). *Escherichia coli* strain JM109 (Promega) was transformed and screened to select plasmids containing inserts, which were sequenced. Gaps in the genome sequence which were not covered by clones derived from the cDNA library, were completed by reverse transcription and PCR primed by oligonucleotides complementary to sequences flanking the gaps. The ends of the molecules were cloned using the RLM-RACE method described by Coutts and Livieratos (2003).

Table 1. EMBL nucleotide database accession numbers of members of the families *Chrysoviridae* and *Totiviridae* used for phylogenetic analysis. Species used in the alignment for the design of the Totioligo primer are in bold.

Virus	Abbreviation	Accession no.
Amasya cherry disease associated chrysovirus	ACDACV	AJ781397
Agaricus bisporus virus 1	AbV 1	CAA64144
Armigeres subalbatus totivirus	AsTV	EU715328
Aspergillus fumigatus chrysovirus	AfuCV	FN178512
Aspergillus mycovirus 1816	AV-1816	EU289896
Botryotinia fuckeliana totivirus 1	BfV1	CAM33265.1
Chalara elegans RNA virus 1	CeRV1	AY561500
***Coniothyrium minitans* RNA virus**	CmRV	AAO14999.1
Cryphonectria nitschkei chrysovirus 1	CnV-1	ACT79255
Drosophila melanogaster totivirus	DTV	GQ342961
***Epichloë festucae* vírus 1**	EfV1	CAK02788.1
Fusarium oxysporum chrysovirus 1	FoCV-1	EF152346
Giardia lamblia virus	GLV	L13218
***Gremmeniella abietina* RNA virus L1**	GaRV-L1	AF337175
Gremmeniella abietina RNA virus-L2	GaRV-L2	AY615210
***Helicobasidium mompa* totivirus 1-17**	HmV-17	BAC81754.1
Helminthosporium victoriae-145S virus	Hv-145S	AF297176
***Helminthosporium victoriae* virus 190S**	Hv-190SV	AAB94791.2
Infectious myonecrosis virus	IMNV	EF061744
Leishmania RNA virus 1-1	LRV1-1	M92355
Leishmania RNA virus 2-1	LRV2-1	U32108
Leishmania RNA virus 1-4	LRV1-4	U01899
Magnaporthe oryzae virus 2	MoV 2	BAF98178.1
Magnaporthe oryzae chrysovirus 1	MoCV1	BAJ15133
Omono River virus-AK4	OMRV-AK4	AB555544
Omono River virus-Y61	OMRV-Y61	AB555545
Penicillium chrysogenum virus	PcV	AF296439
***Saccharomyces cerevisiae* virus-LA**	ScV-L-A	J04692
Saccharomyces cerevisiae virus-L-BC	ScV-L-BC	U01060
Sphaeropsis sapinea RNA virus 1	SsRV-1	AAD11601.1
***Sphaeropsis sapinea* RNA virus 2**	SsRV-2	AAD11603.1
Tolypocladium cylindrosporum virus 1	TcV 1	FR750562
Tolypocladium cylindrosporum virus 2	TcV 2	FR750563
Trichomonas vaginalis virus 2	TVV	AF127178
Ustilago maydis virus H1	UmV-H1	U01059
Verticillium chrysogenum virus	VcV	ADG21213

Northern blotting experiments

Two Northern blot hybridizations were done to determine to which dsRNA element the cDNA contigs assembled belonged. DsRNA extracts from strains 11 and 3398M were electrophoresed in agarose gels, denatured, and transferred to nylon membranes

(Zabalgogeazcoa *et al.,* 1998). Hybridization and detection were done using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

Sequence analyses

Sequence similarity searches in the EMBL virus sequence database were conducted using the FASTA program (Pearson, 1990). For phylogenetic analyses sequence alignments of genes of *T. cylindrosporum* viruses and others of similar sequence were done with ClustalX (Thompson *et al.,* 1997). Phylogenetic analyses of amino acid sequences were done with MEGA software (Kumar *et al.,* 2004). Genetic distances were calculated with the Poisson correction model. Phylogenetic trees were made using the neighbour-joining method, and bootstrap test values were based on 1000 replications.

Virion purification

Strains 3398M, 11, and a monosporic isolate derived from isolate 11 and harbouring only dsRNA1 (11-1L) were cultured over cellophane disks layered on top of PDA plates for three weeks. Twenty grams of each isolate were used to obtain partially purified virus preparations, as well as sucrose gradient purified preparations, using the method described by Jiang and Ghabrial (2004).

To check which dsRNA elements were encapsidated in protein, partially purified virus preparations obtained from strains 3398M and 11 were treated to disrupt virions. Only encapsidated dsRNA, which can be sedimented by high speed centrifugation, should be present in these virus preparations, naked dsRNA would not sediment at the high speed centrifugation used for particle purification (Zabalgogeazcoa *et al.,* 1998). For virion disruption, the partially purified virus preparations were incubated for 20 min at 60 ºC in the presence of 0.1% SDS, and then treated with 1 volume of phenol:chlorophorm (1:1). The aqueous phase was ethanol precipitated, and the presence of dsRNA was checked by electrophoresis.

The molecular weight of proteins present in sucrose-gradient virus preparations obtained from strain 11-1L, harbouring only dsRNA1, was determined in two experiments using SDS-10% polyacrilamide gel electrophoresis. The gels were stained with coomassie blue (Laemmli, 1970).

Electron microscopy

Formvar-coated 300 mesh copper grids were placed on top of 20 µl drops of sucrose gradient virus preparations of TcV1. Excess solution was absorbed from the grids, which were then stained with 2% uranyl acetate, pH 4.5. The grids were examined in a Zeiss 900 transmission electron microscope.

Curing experiments

The antiviral compound ribavirin was used in an attempt to cure dsRNA infected strains. Strains 11 and 3398M were grown under shaking (110 rpm) for 12 days at 24 ºC in two different curing media consisting of PDB containing 80 or 100 µM of ribavirin. The range of biologically active concentrations of this compound is 10 to 100 µM (Parker, 2005). Monosporic isolates of each strain and treatment were obtained as explained before. All monosporic isolates were analyzed for the presence of dsRNA by CF-11 cellulose chromatography. Isolates apparently cured of infection by all mycoviruses were analyzed two additional times.

DsRNA influence in the growth response to temperature

To study the effects of the different viruses in the growth of strains 11 and 3398M at different temperatures, we used isogenic strains harbouring different combinations of the dsRNA elements that originally infected the parental strains 11 and 3398M (Table 2). These isogenic strains were obtained from the transmission experiments in liquid culture without adding ribavirin. Four isolates derived from strain 11 and two derived from 3398M plus the parental strains were analyzed. The eight isolates were cultured in PDA plates at two different temperatures, 22 ºC and 29 ºC. Preliminary tests carried with isolates 11 and 3398M showed that in the conditions used for this study, the isolates did not grow at 32 ºC. Bissett (1983) reported that 22 ºC is the optimal temperature for growth on PDA for all the species of the genus. A 3 x 3 mm block of mycelium was placed in the center of a 9 cm PDA plate which was sealed with Parafilm. Six plates of each isolate were placed in two incubators set at 22 and 29 ºC. The position of the 48 culture plates incubated together at each temperature was randomized at each temperature, and the diameter of the colonies was measured after 21 days.

For each temperature and group of isogenic strains, a one way ANOVA was used to test the statistical significance of differences among the mean diameters of each isolates,

and the LSD procedure was used to separate means; values of $p < 0.05$ were considered significant. Statistica 5.0 (StatSoft, USA) software package was used to conduct these analyses.

Table 2. Monosporic *T. cylindrosporum* isolates employed in studies of influence of dsRNA elements in the radial growth and response to temperature of *T. cylindrosporum*.

Isolate Code	dsRNAs present	Viral infection
[a]**11-6B**	All (dsRNA1-dsRNA6)	TcV1+TcV2+TcV3
11-2B	dsRNA1 + dsRNA2	TcV1+TcV3
11-1L	dsRNA1	TcV1
11-1S	dsRNA2	TcV3
11-0B	None	None
[a]**3398-5B**	All (dsRNA2-dsRNA6)	TcV2+TcV3
3398-1B	dsRNA2	TcV3
3398-0B	None	None

[a]Parental strains.

Effect of temperature on dsRNA concentration

The cellular concentration of the victorivirus EfV1 greatly increases when the fungus is grown at a higher temperature (Romo *et al.,* 2007). An experiment was made to find out if the same occurred with the viruses associated to *T. cylindrosporum*. Three isogenic viral infected isolates derived from strain 11 and one derived from 3398M plus these parental strains (Table 2), were grown at 22 and 29 ºC for a period of 21 days on cellophane disks placed on top of PDA plates. Four plates of each isolate were incubated at each temperature. After this treatment the mycelium was lyophilized and dsRNA was extracted from 0.15 g of mycelium from each isolate. The possible change in the concentration of dsRNA at each temperature was visualized by electrophoresis in 1% agarose gels.

Results

Transmission of dsRNA elements to asexual spores

Two monosporic strains of *T. cylindrosporum*, 11 and 3398M, were used to study the transmission of dsRNA elements to asexual spores. Strain 11 harboured six dsRNA elements (dsRNA1 to dsRNA6), with sizes ranging from 5.1 to 3.1 kbp, strain 3398M harboured five dsRNA elements (dsRNA2 to dsRNA6), and dsRNA1 was missing in this strain (Fig. 1).

In both strains, different dsRNA elements showed different rates of transmission to mitotic spores (Table 3). Among the 43 monosporic isolates obtained from strain 11, dsRNA1 had the highest percentage of transmission, it was present in 81.4% of the isolates, and dsRNA2 was transmitted to 79.1% of the monosporic isolates, alone or together with dsRNA1. DsRNAs 3, 4, 5 and 6 showed the same rate of transmission to mitospores, they were present only in 4.7% of the isolates, and always accompanied by dsRNA1 and dsRNA2. Regarding strain 3398M, no monosporic isolates harbouring its five original dsRNA elements were obtained from the 40 monosporic isolates analyzed. DsRNAs 3, 4, 5 and 6 were not transmitted to any spore progeny. In contrast, dsRNA2 was transmitted to 90% of the monosporic progeny, and four isolates were dsRNA-free.

Table 3. Transmission of dsRNA elements to monosporic strains derived from strain 11, which harbours six dsRNA elements, and strain 3398, carrier of five dsRNA elements. The parental strains were grown in shaking cultures with or without ribavirin, and strains derived from the conidia produced in these cultures were analyzed for the presence of dsRNA.

Parental strain	dsRNA elements in parental strain	dsRNAs present in monosporic strains derived from parental strain	Number of strains/total		
			Natural transmission (no ribavirin)	80 μM ribavirin	100 μM ribavirin
11	dsRNA 1,2,3,4,5,6	All (dsRNA1-dsRNA6)	2/43	0/47	0/48
		dsRNA1 + dsRNA2	27/43	0/47	0/48
		dsRNA1	6/43	0/47	0/48
		dsRNA2	5/43	26/47	38/48
		None	3/43	21/47	10/48
3398M	dsRNA 2,3,4,5,6	All (dsRNA2-dsRNA6)	0/40	0/47	0/44
		dsRNA2	36/40	37/47	41/44
		None	4/40	10/47	3/44

The above results suggest that the six dsRNA elements are the product of a multiple virus infection. Isolates infected only by dsRNA1 or by dsRNA2 were obtained from both strains. However, dsRNAs 3, 4, 5, and 6 did not segregate in conidial progeny. Therefore dsRNA1 could be the genome of a virus and dsRNA2 the genome of another. The four remaining dsRNAs could represent the multipartite genome of a third virus because in addition to be transmitted together, the size of these dsRNAs (3.1-3.7 kbp) is too small to be the genome of an encapsidated monopartite virus.

Nucleotide sequence and organization of the virus genomes

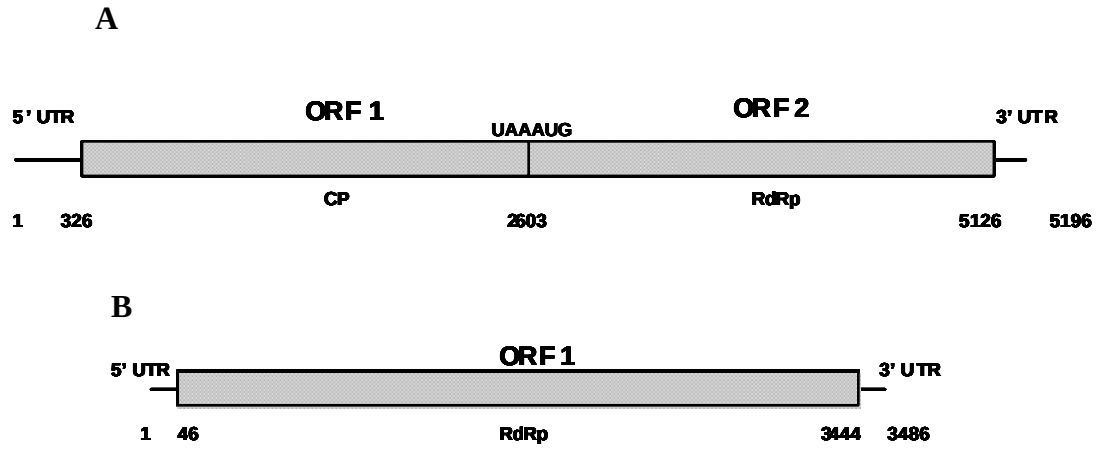
DsRNA purified from strain 11 was used as a template for the synthesis of a cDNA library. Thirty five different cDNA clones were obtained using a degenerate primer, and three contigs were obtained after sequencing and assembling these clones. The use of this oligonucleotide instead of random hexamers improved the success of obtaining cDNA libraries. Northern blot hybridizations using probes made with one clone from each contig showed that two contigs were complementary to dsRNA1, and the other one to dsRNA3. In addition, the hybridizations showed that the sequence of dsRNA3 is similar in strains 11 and 3398M. No cDNA clones of the other four dsRNA elements were obtained. The gap between the two contigs complementary to dsRNA1 was filled using specific primers flanking it, and this experiment was performed three times. Four identical clones of the 5' end and five identical clones of the 3' end of dsRNA1, obtained from two independent RLM-RACE experiments for each terminus, were sequenced. To obtain a complete sequence of dsRNA3, four identical cDNA clones of the 5' end and three identical clones of the 3' end from two independent RLM-RACE experiments of each terminus were sequenced.

The complete sequence of dsRNA1 had 5196 bp, and contained two open reading frames (ORFs) (Fig. 2). ORF1 consists of 2277 bp and encodes a hypothetical 758 amino acid protein (79.9 kDa); ORF2 is 2523 bp long and encodes an 840 amino acid protein (91.2 kDa). Both ORFs are in the same reading frame, the UAA stop codon from ORF1 is directly followed by the AUG start codon of ORF2. No other possible ORFs longer than 350 nucleotides were found in any strand. The complete genome has a GC content of 61%.

The 5' untranslated region (UTR) has 326 bp and a GC content of 59%. The terminus of this region has a GAAAT sequence, similar to the GAAAA motif present in the genomes of *Ustilago maydis* Virus (UmV) and the *Saccharomyces cerevisiae* viruses

ScV-LA and ScV-L BC (Fujimura and Wickner, 1988; Kang *et al.,* 2001). The 3' UTR has a length of 70 bp, a GC content of 64% and the same terminal AUGC sequence as ScV-LA (Wickner, 1996).

Fig. 2. A. Genome organization of *Tolypocladium cylindrosporum* virus 1 (TcV1). The 5196 kbp genome contains two ORFs; ORF1 encodes a putative CP and ORF2 a putative RdRp. **B.** Organization of dsRNA 3, a 3486 bp element which encodes a putative RdRp.



The amino acid sequence deduced from ORF1 of dsRNA1 exhibits a high degree of identity to those of the capsid proteins (CP) of viruses of the family *Totiviridae*, particularly to that of *Botryotinia fuckeliana* virus 1 (BfV1; 61.5%). The C-terminus of this putative CP has an Ala/Gly/Pro-rich region, which occurs in mycoviruses of the *Victorivirus* genus (Ghabrial and Nibert, 2009). The deduced amino acid sequence of ORF2 from dsRNA1 resembled those of RdRps of viruses of the family *Totiviridae*, particularly that of BfV1 (49% identity). The eight conserved motifs of the sequences of RdRps of dsRNA viruses of simple eukaryotes (Bruenn, 1993) were found in the amino acid sequence deduced from ORF2.

The size of dsRNA1 and the genes present on it indicated that this molecule could constitute the genome of a virus belonging to the *Totiviridae* family. We nominated this new virus *Tolypocladium cylindrosporum* virus 1 (TcV1), and its complete genome sequence has been deposited in the EMBL nucleotide sequence database with accession number FR750562.

DsRNA3 had a length of 3486 bp with a 50% GC content, and contained a 3399 bp ORF that encodes an 1132 amino acid protein (127.7 kDa) (Fig. 2). The 5' UTR has 45 bp and 33% GC content. The 3' UTR is 42 bp long and its GC content is 40%. Some sequences similar to those described in the 5' termini of some chrysoviruses were observed in or near the 5' UTR termini of dsRNA3. For instance, a GAUAAA sequence similar to the 5' termini of some chrysoviruses occurred in position 12 (Ghabrial, 2010), and an AAAAAA sequence in position 4 (Jamal *et al.,* 2010). The amino acid sequence deduced from the unique ORF present in dsRNA3 exhibited the highest identity to the RdRp of *Magnaporthe oryzae* chrysovirus 1 (50% identity) (Urayama, *et al.,* 2010), but also resembled other chrysovirus replicases. The eight conserved motifs of the sequences of RdRps of dsRNA viruses of simple eukaryotes (Bruenn, 1993) were also found in the amino acid sequence deduced from the unique ORF present in dsRNA3**.**

No clones were obtained from dsRNA2 but according with the results of the transmission experiment, this dsRNA2 could constitute the complete genome of a virus that was tentatively named TcV3.

Phylogenetic analysis

A phylogenetic analysis based on the amino acid sequence of the CP and RdRp of selected members of the *Totiviridae* and *Chrysoviridae* families and those of TcV1 (dsRNA1) and dsRNA3 was made (Fig. 3). This analysis showed that TcV1 resembles most the mycoviruses included in a clade within the genus *Victorivirus* (Fam. *Totiviridae*). The alignment of the 3' UTR sequences of all eleven known members of the *Victorivirus* genus and TcV1 (dsRNA1), revealed a region of 18 bp that is conserved in most members of the genus, except in *Helicobasidium mompa* totivirus 1-17 (HmV-17) and *Coniothyrium minitans* RNA virus (CmRV) (Fig. 4).

The phylogenetic analysis confirmed that dsRNA3 is closer to the *Chrysoviridae* family than to the *Totiviridae.* Since chrysoviruses have tetrapartite genomes of 2.4-3.6 kbp (Ghabrial *et al.,* 2005b), the fact that dsRNA3 encodes a putative chysovirus RdRp supports the hypothesis of dsRNAs 3, 4, 5 and 6 being the multipartite genome of a virus, and in particular, of a member of the *Chrysoviridae*. We have named this virus *Tolypocladium cylindrosporum* virus 2 (TcV2), and the sequence of dsRNA3, encoding the viral RdRp, has been deposited in the EMBL nucleotide database with accession number FR750563.

Fig. 3. A Phylogenetic tree of viruses of the family *Totiviridae* based on CP amino acid sequences. **B.** Phylogenetic tree based on RdRp amino acid sequences of members of the *Totiviridae* and *Chrysoviridae* families. The unrooted phylogenetic trees are based on the neighbour-joining method. Numbers at nodes represent bootstrap values as percentages estimated by 1000 replicates. The accession numbers of sequences used in the analyses are given in Table 1.

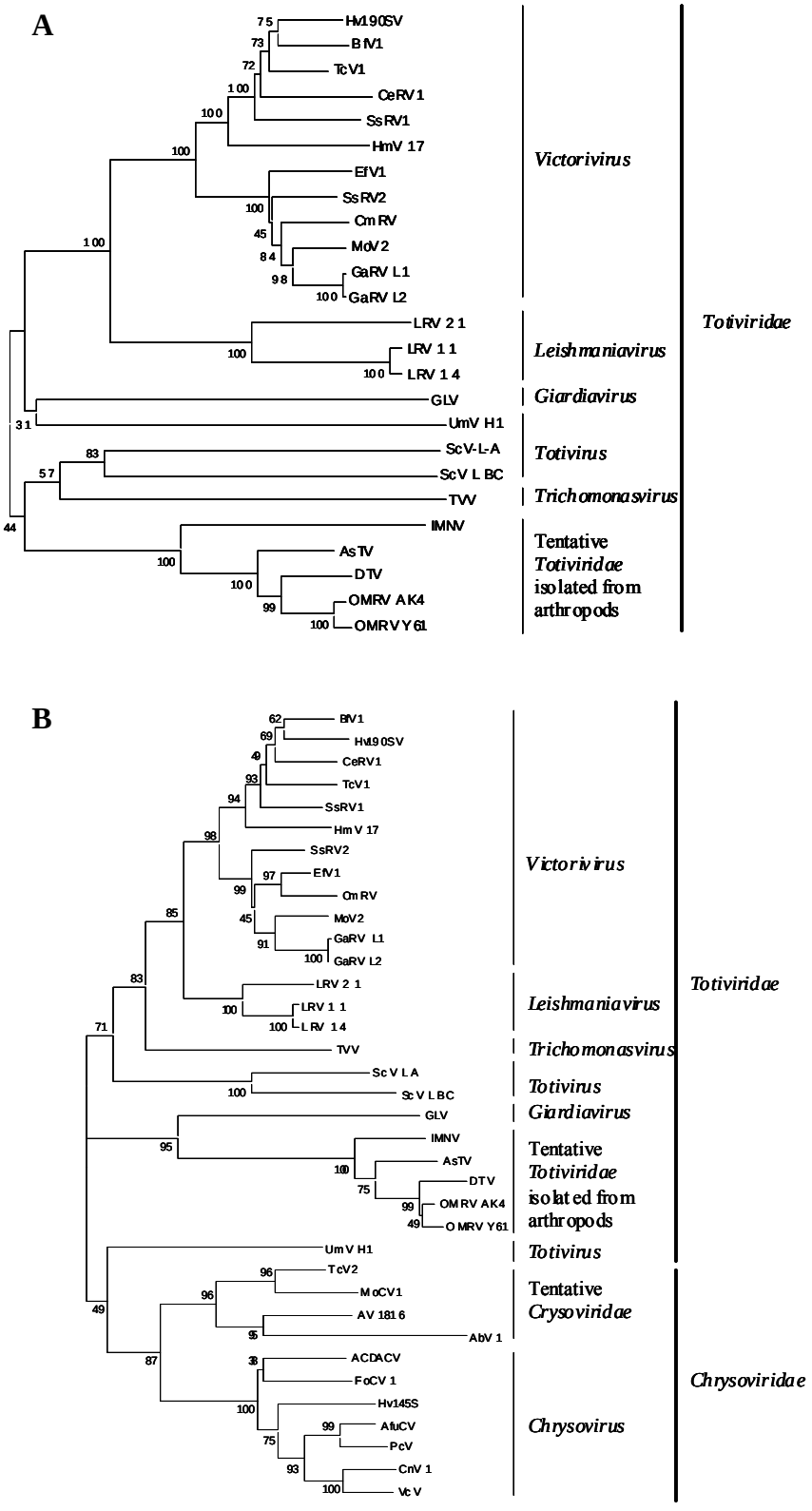
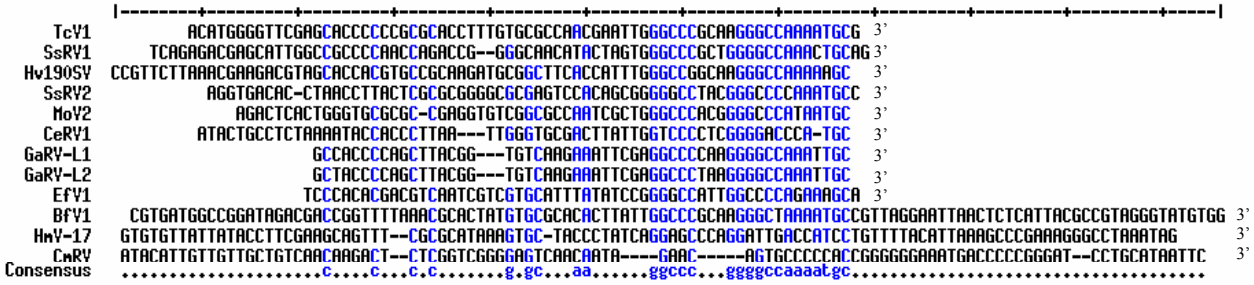


Fig. 4. Alignment of the 3' UTR sequences of eleven members of the *Victorivirus* genus and TcV1. A conserved sequence (blue letters) among members of this genus is indicated.

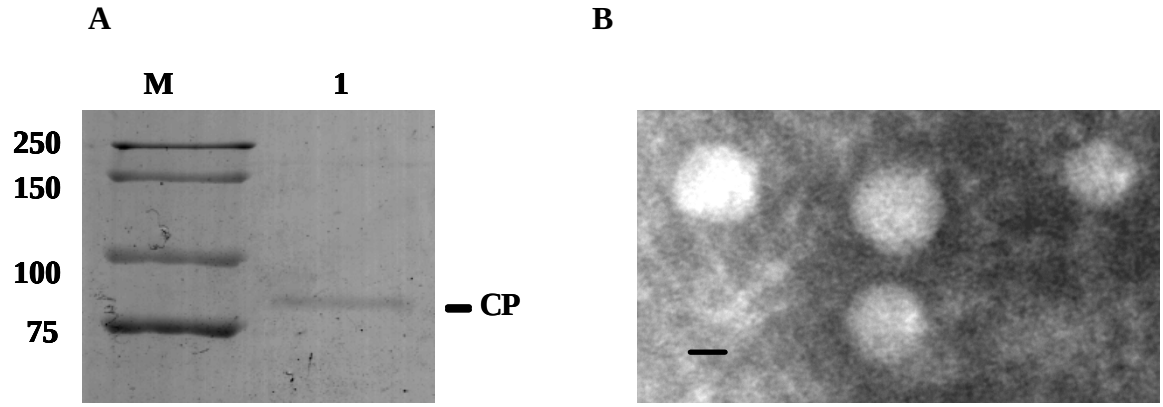


Analysis of virus particles and structural proteins

Partially purified virions from strains 11 and 3398M were disrupted by treatment with SDS and phenol in order to determine if all dsRNAs infecting these strains were encapsidated. All dsRNAs infecting each strain were recovered from the aqueous phase obtained after the disruption treatment. This result suggests that all six dsRNA elements are encapsidated in protein particles.

When a sucrose gradient purified virus preparation of strain 11-1L, harbouring only dsRNA1**,** was examined by transmission electron microscopy (TEM), isometric virus like particles of approximately 50 nm of diameter were observed (Fig. 5).

Fig. 5. **A.** SDS-PAGE analysis of purified TcV1 particles. Lane M, molecular weight marker (kDa); lane 1, SDS-treated TcV1 virions. The structural proteins were visualized by coomassie blue staining. The 79.7 kDa protein purified by electrophoresis (CP) has a molecular weight similar to the one estimated for the hypothetical protein coded by ORF1 (79.87 kDa). **B.** Isometric virus-like particles observed by TEM from a purified virus preparation from *T. cylindrosporum* 11-1L isolate harbouring only dsRNA1. Bar, 25 nm.



A sucrose gradient virus preparation obtained from strain 11-1L, was separated by SDS-PAGE in order to analyze virion proteins. Coomassie blue staining of the gel showed a major polypeptide with an apparent molecular mass of 79.70 kDa (Fig. 5). This size is similar to that expected from the hypothetical protein of 758 amino acids encoded by ORF1 (79.87 kDa). Therefore, ORF1 appears to encode the CP of the virus whose genome is dsRNA1.

Curing dsRNA elements

Different proportions of cured isolates were observed with each ribavirin treatment. The lower concentration (80 μM) was the most efficient for both strains, 44.7% of the monosporic isolates obtained from strain 11 were free of all six dsRNAs, and the rest only harboured dsRNA2. In the absence of ribavirin only 7.0% of the isolates were completely cured (Table 3). In the case of strain 3398M, 21.3% of the isolates were completely cured and the remainder harboured only dsRNA2, in contrast, only 10% of the monosporic progeny of this strain was virus free in the absence of ribavirin.

With the 100 μM ribavirin treatment 20.8% of the isolates derived from strain 11 were completely cured, and the remainder harboured only dsRNA2. At this concentration, only 6.8% of the conidial progeny of strain 3398M were virus-free, and the remainder carried dsRNA2.

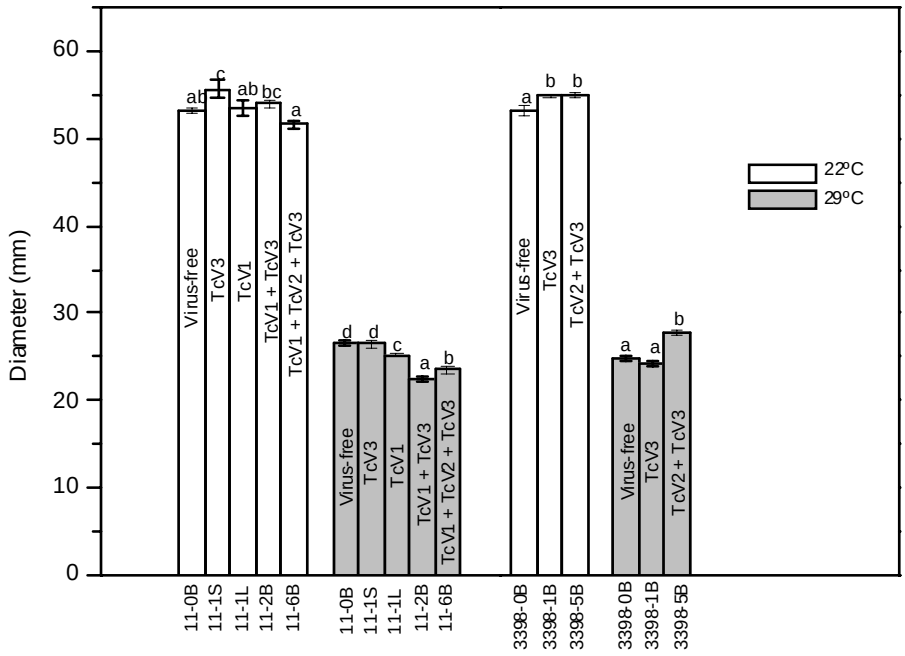
The above results indicate that the effectiveness of ribavirin might be different for each dsRNA element. DsRNA1 was very sensitive to the drug; all the monosporic isolates were free of this mycovirus at both concentrations of ribavirin, but in the absence of ribavirin, the rate of transmission of dsRNA1 to conidia was 81%. In contrast, dsRNA2 was not very sensitive to the action of the antiviral compound. For strain 11 the percentage of transmission of dsRNA2 to conidiospores ranged from 55.5% to 79.2% for isolates treated with 80 and 100 μM concentrations of ribavirin, respectively. For this strain the natural transmission of dsRNA2 to asexual spores was 79.1%, almost the same observed with 100 μM ribavirin. Similarly, the rate of transmission of dsRNA2 in strain 3398M was similar with 100 μM ribavirin (93.2%) or without it (90.0%). With 80 μM ribavirin, the transmission rate of dsRNA2 (78.7%) was somewhat lower than the negative control. The natural transmission of dsRNAs 3, 4, 5 and 6 to conidia was very low in both strains (4.7% - 0%); therefore, it is difficult to evaluate the effect of ribavirin in the elimination of these dsRNAs.

DsRNA influence in radial growth and response to temperature

An experiment was made to test the effects of the different dsRNAs on the radial growth of strains 11 and 3398M at different temperatures. Isogenic strains harbouring different combinations of the two or three possible viruses that originally infected the parental strains 3398M and 11 were obtained from single conidium subcultures, and used in the experiment (Fig. 1; Table 2).

Virus presence did not clearly affect the radial growth of the fungal strains (Fig. 6). At 22 ºC the diameter of strain 11 infected by the three viruses was not significantly different than that of the same strain free of viruses. At this temperature the growth of strain 3398M containing TcV2 and TcV3 was significantly greater than that of its isogenic virus-free version. At 29 ºC, strain 11 isolates harbouring the three viruses, grew less than the virus free strain. The opposite occurred with the 3398M isogenic strains, the one infected by TcV2 and TcV3 grew more than its versions harbouring TcV3 or dsRNA-free. Therefore, the differences observed were strongly influenced by the fungal genotypes, and a plain virus effect is not obvious.

Fig. 6. Average diameter at 22 ºC and 29 ºC after 21 days of growth in groups of isogenic strains harbouring different virus combinations. The isogenic strains were derived from isolate 11, originally infected by TcV1, TcV2, and TcV3, or from isolate 3398M, originally infected by TcV2 and TcV3. In each group of columns, strains sharing the same letter are not significantly different (LSD; $p<0.05$).



High temperature did not seem to affect the cellular concentration of dsRNA in infected isolates. The amount of each dsRNA element observed in extracts obtained from equal weight of mycelium of isogenic strains 3398-5B, 3398-1B, 11-6B, 11-2B, 11-1L, and 11-1S, was similar when the strains were incubated at 29 ºC or at 22 ºC.

Discussion

The existence of mixed virus infections in strains 11 and 3398M could be deduced from the nucleotide sequences obtained, and from the results of the transmission experiments. In liquid cultures, the rate of transmission to conidia was different for each dsRNA element harboured by *T. cylindrosporum* (Table 3). DsRNA1 was completely sequenced and represents the genome of TcV1, a new member of the family *Totiviridae*. TcV1 was transmitted to 81.4% of the monosporic isolates, some of these isolates were infected only by this virus, but others contained additional dsRNAs. DsRNA2 was transmitted to 79.1% of the conidia with or without other dsRNAs, so it could constitute the complete genome of another mycovirus, which we tentatively named TcV3. In contrast, dsRNAs 3, 4, 5 and 6 did not segregate and were always transmitted together to conidia. There are several reasons to think that these four elements might constitute the complete multipartite genome of a member of the *Chrysoviridae* family. DsRNA3 was completely sequenced and encodes an RdRp phylogenetically close to those of the *Chrysoviridae* (Figs. 2 and 3). Members of this virus family have segmented genomes composed of four dsRNA elements with sizes between 2.4 and 3.6 kbp (Ghabrial *et al.*, 2005b). In addition, partial purification of virus particles indicated that the four dsRNAs were encapsidated. Therefore, the four smaller dsRNA elements could constitute the genome of a third virus infecting *T. cylindrosporum*, a hypothetical chysovirus that we have called TcV2. The natural transmission rate of TcV2 to conidia was very low, 4.7% in strain 11 and 0% in 3398M (Table 3). This suggests that perhaps in nature the transmission of TcV2 might not be conidial, but horizontal by cell fusion, or trough cell division. This possibility is supported by the fact that in our laboratory the four dsRNAs have been persistent in cultures after several years of continuous plating, even after cultures were frozen or exposed to high temperatures. Hypocrealean entomopathogens like *T. cylindrosporum* or *B. bassiana* can live in soil, insects, and plants; however, their primary

substrate for conidiation seems to be insects (Meyling and Eilenberg, 2007). Based on this, an alternative explanation for the low rate of transmission observed for TcV2 could be that when the fungus sporulates on insects, or another substrate, the transmission of viruses to conidia could be more efficient than the one we have observed in laboratory cultures.

In all reported cases of naturally ocurring mixed virus infections in fungi (i.e. *Fusarium graminearum, Gremeniella abietina*, *Epichloë festucae, Heterobasidion annosum*) all the viruses infecting a strain were transmitted together to the conidial progeny (Chu *et al.,* 2004; Ihrmark *et al.,* 2002; Romo *et al.,* 2007; Tuomivirta and Hantula, 2005). In contrast, in *T. cylindrosporum* different rates of transmission occurred for each virus, with values ranging from 0% to 90%, depending on the virus. To our knowledge this is the first report of differential transmission rates among viruses in naturally occurring mixed infections.

The dsRNA1 molecule infecting *T. cylindrosporum* has characteristics of a complete virus genome. We have denominated this virus TcV1, and it is the first virus infecting an entomopathogenic fungus whose genome has been sequenced. The genome size (5196 bp) and organization of TcV1 (Fig. 2), as well as its virion morphology is typical of the *Totiviridae* family, whose members have nonsegmented dsRNA genomes of 4.6-6.7 kbp coding for a CP and an RdRp, and are encapsidated in isometric particles of ca. 40 nm (Ghabrial, 2010; Wickner *et al.,* 2005). The phylogenetic analysis and some characteristics of TcV1 allowed ascribing this virus to the *Victorivirus* genus (Fig. 3). Like other victoriviruses, TcV1 infects a filamentous fungus, has a Pro/Ala/Gly-rich region near the C-terminus of the CP, and has 5' and 3' UTRs with sizes similar to those from the genus (Ghabrial and Nibert, 2009). Additionally, the alignment of the 3' UTRs of members of the genus showed a highly conserved sequence of 18 bp in this region (Fig. 4). Several victorivirus genomes, including that of TcV1, end with an AUGC 3' motif. This motif and a preceding stem loop, also predicted in TcV1, have an important role in the replication of *Saccharomyces cerevisiae* totivirus ScV-LA (Wickner, 1996), and could have a similar function in victoriviruses. Nevertheless, TcV1 differs from other members of the *Victorivirus* genus in the fact that its two ORFs are in the same reading frame and do not overlap, as occurs with most members of the genus. In TcV1 the UAA stop codon of ORF1 is immediately followed by the AUG start codon of ORF2. A study performed with a mutant of the mycovirus CHV1-EP713 proved that the non existence of an overlap between ORF1 and ORF2 does not affect the translation of ORF2 (Guo *et al.,* 2009).

Therefore, TcV1 may also follow a coupled termination reinitiation mechanism of translation, which is typical of victoriviruses (Ghabrial and Nibert, 2009).

As it happens with many mycoviruses, the ones from *T. cylindrosporum* did not have an obvious effect in their host fungus. No clear relationship between the presence of viruses and the radial growth of *T. cylindrosporum* strains at 22 or 29 ºC was observed (Fig. 6). In addition, the cellular concentration of the viruses did not seem to increase when fungi were incubated at high temperature, as it occurs with the victorivirus EfV1 (Romo *et al.,* 2007).

Ribavirin is a nucleoside analog that induces mutations in RNA viral genomes, and has demonstrated a broad spectrum antiviral activity (Parker, 2005). For example, when tested in potyvirus-infected potatoes, about 50% of the treated plants were cured (Mahmoud *et al.,* 2009). This substance has also been used in therapy against human RNA viruses such as hepatitis virus C or herpes virus (Parker, 2005). However, it was not effective against a mitovirus infecting *Chalara elegans* (Park *et al.,* 2006a). In this work virus-infected strains of *T. cylindrosporum* could be cured using ribavirin, a concentration of 80 or 100 μM of ribavirin in the culture medium of strain 11 resulted in the cure of 100% of the monosporic isolates from TcV1. In the absence of ribavirin this mycovirus showed a high rate of transmission to asexual spores (81.4%), so the drug seems to be responsible for the elimination of TcV1. To our knowledge, this has been the first time in which ribavirin is tested succesfully against fungal viruses. However, ribavirin showed different effectiveness for each mycovirus infecting *T. cylindrosporum*. Its effectivity against TcV2 and TcV3 was not obvious. This difference in the response of different viruses to the drug might explain why ribavirin did not work against a *Chalara elegans* virus (Park *et al.,* 2006a).

Viruses infecting insects from the *Culex* and *Drosophila* genera have been recently described, and seem to be members of the *Totiviridae*, the same family as TcV1 (Isawa *et al.,* 2011; Wu *et al.,* 2010). This hypothetical virus link between insects and entomopathogenic fungi is very interesting because viruses might have flowed between kingdoms.

In conclusion, this study presents the first reported sequences for mycoviruses infecting an entomopathogenic fungus, and adds a new member to the genus *Victorivirus*, TcV1. In addition, curing and transmission experiments indicate that the *T. cylindrosporum* strain 11 is infected by three different viruses: TcV1 (dsRNA1); TcV2 (dsRNAs 3, 4, 5, 6),

a hypothetical member of the *Chrysoviridae* family, and TcV3 (dsRNA2), an unknown virus with a genome or a replicative form of approximately 4.2 kbp. Strain 3398M is only infected by TcV2 and TcV3. The size of the genomes of these three viruses is similar to that of some dsRNA elements reported in other hypocrealean entomopathogenic fungi such as *M. anisopliae*, *M. flavoviride*, *B. bassiana*, *P. amoenoroseus,* or *P. fumosoroseus* (Bidochka *et al.,* 2000; Dalzoto *et al.,* 2006; Inglis and Valadares-Inglis, 1997; Melzer and Bidochka, 1998; Souza Azevedo *et al.,* 2000).

In addition, this is the first report of the successful use of ribavirin to cure fungal viruses. This drug could be very helpful to cure infected isolates, and therefore to study the effects that viruses produce in their fungal hosts, something which is largely unknown in spite of the ubiquity of viruses in the fungal kingdom.

Finally, this study constitutes the basis for further studies about the effects that mycoviruses could produce in *T. cylindrosporum*. If mycoviruses affect the pathogenicity of the fungus against insects or ticks; if these viruses are involved in the endophytic capability of *Tolypocladium*; or if they affect the production of secondary metabolites produced by the fungus, such as cyclosporine A, or fumonisins (Fritsche *et al.,* 2004; Mogensen *et al.,* 2011), would be interesting points to be studied in the future.

Acknowledgements

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CAPITULO IV. Capacidad endofítica del hongo entomopatógeno *Tolypocladium cylindrosporum* en plantas de tomate y judía

Antecedentes

Especies pertenecientes a géneros de hongos entomopatógenos como, *Beauveria*, *Paecilomyces*, *Cladosporium*, *Metarhizium*, *Lecanicillium* o *Tolypocladium* han sido descritas como endofitos en diferentes especies de plantas monocotiledóneas y dicotiledóneas (Vega *et al.*, 2008; Sánchez *et al.*, 2011). Adicionalmente, algunos de estos hongos entomopatógenos han sido inoculados artificialmente en diferentes especies de plantas, con el objeto de ser usados como potenciales agentes de control biológico contra plagas de insectos fitófagos o incluso contra patógenos de plantas. Así, *B. bassiana* produce metabolitos bioactivos que limitan el crecimiento de algunos patógenos de plantas (Ownley *et al.*, 2010). *B. bassiana* ha sido descrito como endofito en cacao, maíz, plántulas de café y plátano, amapola, patata, palmera datilera o pino blanco, después de la inoculación artificial de dichas plantas con suspensiones concentradas de conidios (Ownley y Griffin, 2008).

Algunas de las cepas de *T. cylindrosporum* analizadas en los capítulos anteriores fueron aisladas originalmente como endofitos de gramíneas. En este capítulo se estudió la capacidad endofítica de *T. cylindrosporum* en otros dos hospedadores, tomate y judía. Adicionalmente, como disponíamos de cepas isogénicas del hongo infectadas por TcV1 y libres de infección, también se estudió el efecto de TcV1 en la capacidad endofítica de *T. cylindrosporum*.

Objetivos

1. Estudio de la capacidad endofítica de *T. cylindrosporum* en plantas de tomate y judía.

2. Estudio de la influencia de TcV1 en la capacidad endofítica de *T. cylindrosporum* en plantas de tomate y judía.

Resultados

1. Establecimiento de *T. cylindrosporum* como endofito en plantas de tomate y judía

Las dos cepas isogénicas de *T. cylindrosporum* usadas en el bioensayo, 11-1L, infectada por TcV1, y 11-0BR libre de infección, infectaron plantas de tomate y judía cuando estas fueron inoculadas con soluciones de conidios de 1 x 10^8 conidios/ml. Ambas cepas se establecieron como endofitos, ya que las dos fueron recuperadas de hojas de tomate y judía cinco semanas tras su inoculación.

Este resultado indica también que *T. cylindrosporum* es una especie generalista, ya que fue originalmente aislada de gramíneas y ha sido inoculada con éxito en plantas dicotiledóneas como el tomate y la judía.

2. Influencia de TcV1 en la capacidad endofítica de *T. cylindrosporum*.

No se encontró un efecto significativo del virus en la capacidad endofítica de las dos cepas de *T. cylindrosporum* empleadas en el bioensayo. No obstante, se observó una interacción significativa entre la presencia de virus y la especie vegetal hospedadora, así, las cepas de *T. cylindrosporum* infectadas por TcV1 entraron mejor en plantas de judía. Por el contrario, en tomate las cepas libres de infección por el micovirus fueron las que mejor entraron.

Endophytic establishment of the entomopathogenic fungus *Tolypocladium cylindrosporum* in tomato and bean plants

Noemí Herrero Asensio, Salud Sánchez Márquez, Iñigo Zabalgogeazcoa

Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain

Abstract

The purpose of this work was to test if two strains of *T. cylindrosporum*, originally isolated as endophytes from grasses could be artificially inoculated, and behaved as endophytes in tomato or bean plants. *T. cylindrosporum* strains 11-1L and 11-0BR were reisolated from leaf fragments of both plant species up to 35 days after their inoculation with a concentrated suspension of 1 x 10^8 conidia/ml. Both fungal strains were isogenic, differing only in the mycoviral infection by the *Victorivirus* TcV1 harboured by 11-1L, strain 11-0BR was virus-free. The influence of TcV1 in the endophytic capability of *T. cylindrosporum* was also evaluated. A significant interaction was observed among the mycovirus infection and the capability of each strain to infect each plant host. Virus infected *T. cylindrosporum* strain 11-1L penetrated better in bean plants than the virus-free strain (11-0BR). The opposite tendency was observed in tomato plants, where the virus free strain penetrated better than the infected strain.

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Introduction

Endophytes are a group of fungi occurring inside plant tissues without causing any apparent symptoms to them. Nonetheless, several roles have been attributed to endophytic fungi, like providing resistance to abiotic stresses (Rodríguez *et al.,* 2009), protection against plant pathogenic nematodes and fungi (Elmi *et al.,* 2000; Wicklow *et al.,* 2005; Zabalgogeazcoa, 2008), or protection against herbivorous insects (Vega *et al.,* 2008). The

study of this group of fungi was greatly stimulated with the discovery of *Neotyphodium* endophytes, which were found to be responsible for the toxicity of some species of grasses used for animal production (Bacon *et al.*, 1977; Fletcher and Harvey, 1981). Surveys of endophytes in other plant families showed that *Neotyphodium* species and their *Epichloë* teleomorphs are associated to a small number of grass species. However, these studies revealed a large number of fungal species infecting asymptomatically many plant species, and at the present time, no endophyte-free plant species has been reported (Arnold, 2007; Hyde and Soytong, 2008).

Species belonging to entomopathogenic genera like, *Beauveria*, *Paecilomyces*, *Cladosporium*, *Metarhizium*, *Lecanicillium* or *Tolypocladium* have been reported as endophytes in different monocotyledonous and dicotyledonous plant species (Sánchez Márquez *et al.*, 2011; Vega *et al.*, 2008). Some of these entomopathogenic fungi have been artificially inoculated in different plant species in order to be used as biological control agents against plant pests, or even against fungal pathogens of plants, since species as *B. bassiana* are known to produce an array of bioactive metabolites that limit the growth of some fungal plant pathogens (Ownley *et al.*, 2010). According to this, *B. bassiana* has been successfully established as an endophyte in cocoa, corn, coffee seedlings, opium poppy, potato, banana, date palm or western white pine, after artificial inoculation with concentrated suspensions of conidia (Ownley and Griffin, 2008).

Tolypocladium cylindrosporum is an entomopathogenic fungus that has been isolated as an endophyte from grasses like *Festuca rubra* and *Holcus lanatus* (Sánchez Márquez *et al.*, 2010), and also from *Theobroma cacao* trees (Hanada *et al.*, 2010). *Tolypocladium* strains isolated from grasses have been reported to harbour mycoviruses (Herrero *et al.*, 2009; Herrero and Zabalgogeazcoa, 2011). The relationship between a virus and its fungal host is similar to that of an endophyte and its host plant; mycoviruses rarely cause obvious symptoms on their hosts. Nevertheless, in some cases mycoviruses affect their hosts causing hypovirulence in some plant pathogens, or disease in *Agaricus bisporus* mushrooms (Ghabrial and Suzuki, 2009; Nuss, 2005; Romaine and Goodin, 2002). A case of a mutualistic mycovirus which improves the thermal tolerance of a plant-fungal endophyte association has been also reported (Marquez *et al.*, 2007).

The main objective of this work was to test the endophytic capability of two strains of the entomopathogenic fungus *T. cylindrosporum*, first isolated as grass endophytes, in two plant species of agricultural interest, tomato and bean. The two *T. cylindrosporum*

strains employed in the bioassay were isogenic but differed in the presence of the mycovirus TcV1. This way, another objective of this work was to investigate if the mycovirus affected the capability of *T. cylindrosporum* to behave as an endophyte in tomato and bean plants.

Materials and Methods

Fungal strains and inoculum preparation

Two isogenic strains of *T. cylindrosporum*, 11-1L and 11-0BR, were used in this study. Both were monosporic isolates derived from the *T. cylindrosporum* monosporic strain 11, which was originally isolated as an endophyte from the grass *Festuca rubra* in natural grasslands of western Spain (Sánchez Márquez *et al.*, 2010). Strain 11-1L is infected by TcV1, a member of the family *Totiviridae* and genus *Victorivirus*, and strain 11-0B is an isolate cured of viral infection with the antiviral ribavirin (Herrero and Zabalgogeazcoa, 2011).

To obtain conidial suspensions, each strain was grown on potato dextrose agar (PDA) Petri plates at room temperature (22–25 ºC) in the dark. Conidia from 20 day old cultures were released from the mycelium with a glass rod, after adding 5 ml of sterile water containing 0.01% Tween 80 to each plate. The conidial suspensions from the plates were collected and centrifuged at 5000 x g for 10 min. The pellets were resuspended in sterile water and the concentration of conidia was estimated with a Bürker chamber (Zabalgogeazcoa *et al.*, 2008). To prepare these suspensions from *T. cylindrosporum*, on average we obtained about 1.5 x 10^7 spores from each gram of fresh mycelium. A 20 day culture on a PDA Petri plate contained about 1.5 g of mycelium. Suspensions of 1 x 10^8 conidia/ml in water containing 0.01% Tween 80 were used for the inoculations.

Plant material and inoculation

Two species of commercial interest, bean (cv. Blanca Planchada) and tomato (cv. Tres Cantos), were chosen for the study. Seeds from both plant species were surface sterilised with a 2 min immersion in commercial bleach (5% active chlorine), and rinsed three times with sterile water. Individual seeds were germinated in 16 cm diameter pots filled with a 50/50 mixture of peat and perlite substrate, which was previously sterilized in an oven at 100 ºC for 72 hours. The tomato and bean plants were grown in a greenhouse at

temperatures that oscillated from 14 to 27 ºC between day and night at an ambient humidity. Tomato plants grew for 30 days, and bean plants for 15 days before the inoculation.

Two ml of the conidial suspension (1 x 10^8 conidia/ml) or of the sterile 0.01% Tween 80 control solution were applied to each tomato or bean plant by spraying with a manual atomizer. The four first true leaves, in the case of tomato plants, and the first two true leaves in the case of bean plants, were sprayed with the conidial suspension. Twenty five plants of each species were inoculated with each fungal strain, and another 25 plants of each species were mock inoculated with the control solution,

Determination of endophytic infection

Infection by the two isogenic strains of *T. cylindrosporum*, 11-1L and 11-0BR, in bean and tomato plants was determined at 3, 7, 14, 21 and 35 days post inoculation (dpi), by fungal reisolation. To isolate the fungi from the plants, small square leaf pieces, measuring about 5 mm per side were cut from the inoculated leaves of five plants per treatment at each sampling date. The leaf pieces from each plant were surface sterilized with a solution of 20% commercial bleach (1% active chlorine) for 10 minutes, and rinsed in sterile water. Thirty two leaf fragments from each plant were placed in two Petri plates with PDA containing chloramphenicol (200 mg/l), and the Petri plates were incubated at room temperature (22–25 ºC) in the dark. The growth of *T. cylindrosporum* from each leaf piece was recorded for 30 days after the sampling. The same procedure was followed with the control plants.

To check if the fungus moved systemically, tomato and bean leaves formed after the inoculation were also processed for the reisolation of *T. cylindrosporum* at the 21 and 35 day sampling intervals. In this case only one plate containing sixteen pieces of leaves from each plant species and treatment were analyzed. Stems and roots were also checked for *T. cylindrosporum* infection in the 35 sampling interval. Stem fragments were processed as above described, and root pieces were surface-disinfected by means of a 5 minute rinse with ethanol, followed by treatment with a 1% active chlorine solution for 15 minutes, 2 minutes in ethanol, and a final rinse in sterile water (Bills, 1996). One plate containing sixteen pieces of root or stem from each plant was analyzed for each plant species and fungal treatment.

To test whether the disinfection methods used were effective in eliminating surface fungi, imprints of leaf fragments were made by pressing them against the surface of some PDA plates which were incubated without plant parts. The plates were periodically observed to determine if fungi emerged from the prints (Schulz *et al*., 1998).

Morphological characteristics were used to identify *T. cylindrosporum* colonies, although the fungal identification was completed with the molecular identification of some isolates of the reisolated fungi by means of the nucleotide sequence of their ITS1-5.8S rRNA-ITS2 region (Sánchez Márquez *et al.,* 2007).

Statistical analysis

For statistical analysis the percentage of leaf pieces showing positive fungal isolation from each plant were square root transformed. The normality of the transformed data was tested using a Kolmogorov-Smirnov test. Differences in the percentage of infected leaf pieces between fungal treatments and host plant species at each sampling interval (3, 7, 14, 21 and 35 dpi) were analysed using a three-way ANOVA. The control treatment was not included in the statistical analysis.

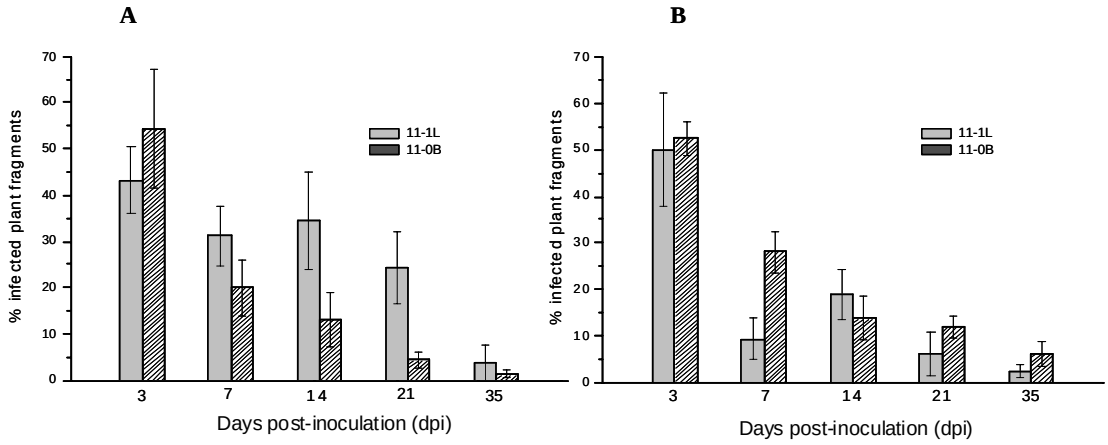
Results

Both isogenic strains of *T. cylindrosporum*, 11-1L, infected by TcV1, and virus-free 11-0BR were able to penetrate tomato and bean plants when sprayed with suspensions of 1 x 10^8 conidia/ml (Fig. 1). No plants of bean or tomato showed any symptom after being inoculated with the fungal strains. *Tolypocladium* mycelium started to emerge from pieces of tomato and bean leaves about eight days after plating the leaf fragments in PDA plates (Fig. 2). Except for a few isolates of other endophytic species, all reisolated fungi were identified morphologically and molecularly as *T. cylindrosporum.*

ANOVA showed that the infection by the TcV1 mycovirus did not have a significant effect in the success of both strains penetrating tomato or bean plants ($F_{1, 80}$ = 0.25; n.s.). Similarly, the mean percentage of infected leaf pieces obtained from bean or tomato plants was not significantly different between both plant species ($F_{1, 80}$ = 0.45; n.s.). In contrast, significant differences in the percentage of infected leaf pieces occurred at the different sampling dates ($F_{4, 80}$ = 33.34; $p<0.05$). In bean and tomato plants, the percentage

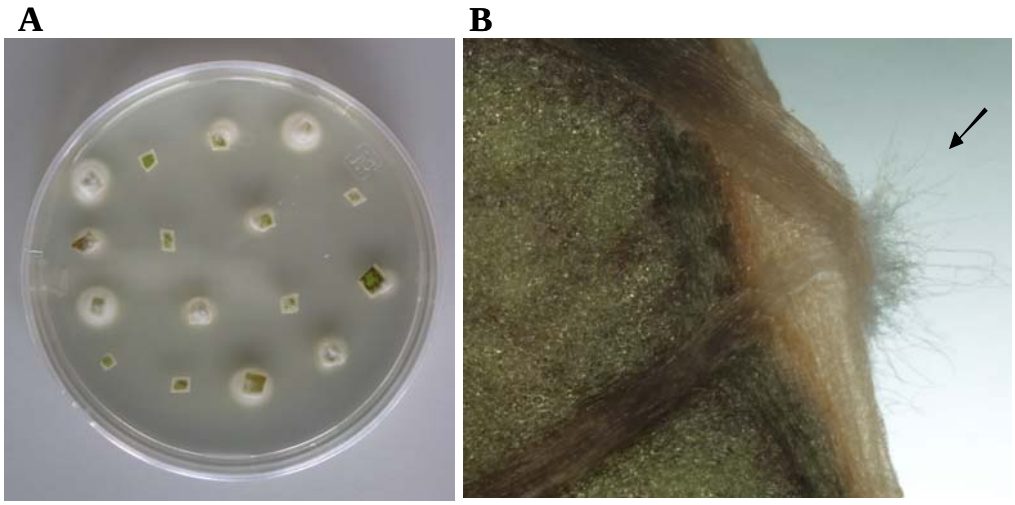
of infected leaf pieces decreased as sampling dates post inoculation increased (Fig. 1). However, in both species the decrease with time was linear for those treated with strain 11-0BR, but for those treated with the TcV1 infected strain there was an increment at 14 days relative to the previous date.

Fig. 1. Percentage (± standard error) of infected plant fragments of (**A)** bean and (**B**) tomato leaves at 3, 7, 14, 21 and 35 dpi.



A significant interaction between plant species and fungal strains was detected ($F_{1, 80}$ =11.87; p<0.05). According to this the TcV1-infected strain infected a greater amount of bean leaf surface than the virus free strain, and the opposite occurred in tomato, where the virus free strain was more effective infecting this host (Fig. 1).

Fig. 2. A. Petri plate with pieces of bean leaves which were sprayed 21 days before with a fungal suspension of 1 x 10^8 conidia/ml showing growth of *T. cylindrosporum* strain 11-1L. **B.** Hyphae of strain 11-0BR emerging from a piece of bean leaf inoculated 21 days before



Both *T. cylindrosporum* strains were reisolated from leaves formed after the inoculation of bean and tomato, 3.75% of infected leaf fragments with strain11-1L, and 1.25% with 11-0BR were obtained at 21 dpi. No fungi were recovered from these leaves at 35 dpi. However, at the 35 day interval *Tolypocladium* was reisolated from stems of tomato (1.25% of infected stem fragments with 11-1L and 2.5% with 11-0BR) and bean plants (1.25% of infected stem fragments with 11-1L). No *T. cylindrosporum* was isolated from control plants.

Discussion

The two strains of *T. cylindrosporum* used in this bioassay (11-1L and 11-0BR) were able to penetrate successfully in tomato and bean plants, and to our knowledge, this is the first report of the endophytic establishment of *T. cylindrosporum* following foliar application of conidia. The endophytic colonisation of corn and opium poppy following foliar inoculation with conidia of *B. bassiana* has been reported (Wagner and Lewis, 2000; Quesada-Moraga *et al.,* 2006a). However, in the present work *T. cylindrosporum* was reisolated from the plants for a longer period of time after the inoculation than in the above works.

The *Tolypocladium* strains that we used were obtained from the grass *Festuca rubra* (Sánchez Márquez *et al.,* 2011). The fact that both strains can penetrate and behave as endophytes in bean and tomato indicates that they behave as host generalists. In contrast with these results, an endophytic strain of *Tolypocladium* spp. isolated from cocoa plants could not be successfully reinoculated in its original host (Hanada *et al.,* 2010).

Although the percentage of isolation of *T. cylindrosporum* decreased significantly with each sampling interval, there was a case in which the percentage of infected leaf pieces did not decrease with the sampling interval, and at 14 dpi strain 11-L1 was reisolated with higher frequency than at 7 dpi in both plant species (Fig. 1). Nonetheless, the decrease in the reisolation of *T. cylindrosporum* from leaves of tomato and bean plants with increasing time could be related to a defensive response from the plant. The increase observed at 14 dpi could be due to an initial success of the fungi in the colonization of the plant before a defence response became effective.

The results obtained do not demonstrate the systemic movement of the fungus in bean or tomato plants. A small number of infected fragments from newly formed leaves were observed, and these could be the result of local infection instead of systemic colonization. Further studies could be made in order to test if using other inoculation methods, the systemic colonization of plants by *T. cylindrosporum* can occur. Other inoculation procedures, most of them applied to *B. bassiana,* have been described in the literature. These methods involved dressing seeds with conidia, dipping roots or rhizomes in conidial suspensions, injecting conidia in rhizomes or stems, or even applying conidia to plant substrates (Akello *et al.,* 2007; Gómez-Vidal et al., 2006; Posada and Vega, 2006; Quesada-Moraga *et al.,* 2006a).

The two *T. cylindrosporum* strains used in this work were isogenic, but they differed in the presence of a mycovirus, TcV1. A significant tendency was observed, the TcV1 infected fungal strain appeared to better penetrate bean plants. In contrast, the virus-free strain seemed to be more successful entering in tomato plants. This suggests that the presence of the virus could be favourable for the fungus in one host species, and unfavourable in another host species. If we would have done this study only with one host, for example with bean plants, we would have concluded that TcV1 produces a positive effect in the endophytic capability of *T. cylindrosporum*, and we would have arrived to the opposite conclusion using tomato as the unique host.

In conclusion, this study demonstrates that *T. cylindrosporum* strains originally isolated as endophytes from the grass *Festuca rubra* were able to establish an endophytic relationship with other plant species than its original host. Therefore, *T. cylindrosporum* could be used for the biological control of invertebrate herbivores or even for the control of plant pathogens, as have been proposed for other entomopathogenic fungi (Ownley *et al.,* 2010). *T. cylindrosporum* has several characteristics that make it suitable for this purpose, like having wide host range, being pathogenic to several insect taxa, and to other arthropods, and its cultures sporulate abundantly, being its spores persistent in storage (Herrero *et al.,* 2011).

CAPITULO V. Caracterización molecular y distribución de micovirus en el hongo entomopatógeno *Beauveria bassiana* en España y Portugal.

Antecedentes

Este último capítulo se centró en el estudio de los micovirus asociados a la especie endofítica y entomopatógena *B. bassiana*. *B. bassiana* es uno de los hongos con mayor potencial como agente de control biológico de insectos fitófagos, de ácaros e incluso de patógenos vegetales (Kaaya y Hassan, 2000; Meyling y Eilenberg, 2007 Ownley *et al.,* 2010). Además ha sido aislado de forma natural como endofito de diversas especies vegetales, e inoculado artificialmente con éxito en otras tantas especies de interés agronómico (Ownley y Griffin, 2008; Vega *et al.,* 2008). Por otro lado, infecciones por micovirus han sido descritas en este hongo entomopatógeno pero ninguno de ellos ha sido secuenciado e identificado hasta el momento (Castrillo *et al.,* 2004; Dalzoto *et al.,* 2006; Melzer y Bidochka, 1998).

En este capítulo se describe un estudio de la incidencia de micovirus en 85 cepas del hongo recogidas principalmente de suelos, filoplano de encina y de plantas adventicias asociadas, así como del interior de hojas de gramíneas, en España y Portugal.

Objetivos

1. Estudio de la incidencia, diversidad y distribución de elementos de dsRNA en aislados de *B. bassiana* recogidos en diferentes hábitats de España y Portugal.

2. Caracterización molecular e identificación de micovirus asociados a *B. bassiana.*

Resultados

1. Incidencia de micovirus en cepas de *B. bassiana* de áreas cultivadas y naturales de España y Portugal

Un total de 85 aislados de *B. bassiana* obtenidos en diferentes hábitats (suelo, filoplano de encina y de las plantas adventicias asociadas, e interior de hojas de gramíneas) en España y Portugal fueron analizados. 50 de los aislados analizados (58.8%) albergaban elementos de dsRNA.

Las incidencias de micovirus fueron también analizadas por separado de acuerdo con el hábitat y la extensión del área donde los hongos fueron aislados. El 53.7% de los 54 aislados de *B. bassiana* obtenidos de suelos en diversas zonas de España y Portugal albergaron elementos de dsRNA. La incidencia de micovirus encontrada en este grupo fue la más baja, mientras que el 68.8% de los 16 aislados de *B. bassiana* muestreados en un mismo punto geográfico de suelo, filoplano de encina y de las plantas adventicias asociadas a una encina fue la más alta. El 66.7% de las quince cepas endofíticas aisladas de gramíneas resultaron infectados por virus. No obstante, todos los porcentajes de incidencia de micovirus obtenidos entre estos tres grupos analizados de *B. bassiana* fueron bastante elevados y a su vez muy parecidos entre sí.

2. Diversidad y distribución de micovirus en el hongo entomopatógeno *B. bassiana*

Una elevada diversidad de micovirus fue encontrada entre las 85 cepas de *B. bassiana* analizadas en este estudio de incidencia. Así, 28 perfiles de dsRNA diferentes fueron encontrados entres las 50 cepas infectadas. El tamaño de los dsRNAs detectados varió entre 1 y 6 kbp, mientras que el número de elementos de dsRNA por aislado estuvo entre 1 y 11. Las características en tamaño y número de los dsRNAs detectados fueron coincidentes con las de genomas de micovirus. De los perfiles de dsRNA detectados se pudieron deducir genomas multipartitos, ya que algunas combinaciones de dsRNAs se repitieron entre aislados. De igual forma, infecciones mixtas pudieron deducirse, ya que algunos elementos de dsRNA o bloques de dsRNAs se encontraron solos infectando a un aislado o en compañía de otros dsRNAs infectando a otros aislados. No se encontró ninguna relación entre determinados perfiles de dsRNA y el hospedador o la localidad donde los aislados de *B. bassiana* fueron muestreados.

Por otro lado, la diversidad de micovirus encontrada no dependió del tamaño del área de muestreo, así se encontró una diversidad similar en un mismo punto de muestreo, bajo una única encina, que en un área de muestreo que comprendía toda la península Ibérica y las islas Canarias y Baleares. No obstante, la diversidad encontrada entre los aislados endofíticos de *B. bassiana* fue muy baja, encontrándose sólo dos perfiles de dsRNA entre los diez aislados infectados por micovirus muestreados en diferentes localidades españolas. Ahora bien, esta elevada diversidad de micovirus encontrada entre los aislados de *B. bassiana* analizados en este estudio se basó en las diferencias encontradas entre los distintos perfiles de dsRNA detectados. No obstante, una hibridación usando como sonda un área del genoma de uno de los virus detectados en este trabajo, BbRV1, reveló que los 13 elementos de dsRNA con el mismo tamaño que BbRV1 detectados en el estudio de incidencia, no se correspondía con la misma especie de micovirus. Lo cual hace pensar en que la diversidad de especies encontrada entre los aislados de *B. bassiana* según los perfiles de dsRNA detectados, podría ser mayor de lo que se había pensado en un principio, ya que un igual perfil de dsRNA no tiene por que corresponderse con la misma especie de micovirus.

3. Caracterización molecular e identificación de micovirus asociados a *B. bassiana*

El micovirus más común encontrado entre los aislados de *B. bassiana* analizados en este estudio de incidencia de micovirus fue totalmente secuenciado e identificado. El genoma completo de este virus tiene 5228 bp y dos ORFs, ORF1 que codifica una CP y ORF2 que codifica una RdRp (Apéndice 3). Un análisis filogenético basado en las secuencias de aminoácidos de RdRps y CPs de otros micovirus de la familia *Totiviridae* y aquellas del virus de *B. bassiana*, indicó que este virus pertenece al género *Victorivirus* (Familia: *Totiviridae*), y ha sido denominado *Beauveria bassiana* RNA virus 1 (BbRV1).

Molecular characterization and distribution of mycoviruses in the entomopathogenic fungus *Beauveria bassiana* in Spain and Portugal

Noemí Herrero[1], Encarna Dueñas[1], Enrique Quesada Moraga[2] and Iñigo Zabalgogeazcoa[1]

1. Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC). Cordel de Merinas 40-52, 37008 Salamanca, Spain.
2. Departamento de Ciencias y Recursos Agrícolas y Forestales, Universidad de Córdoba, Edificio C4 Celestino Mutis, Campus Rabanales, 14071 Córdoba, Spain.

Abstract

A study of incidence showed that mycoviruses are widespread among *B. bassiana* isolates collected from different habitats in Spain and Portugal, 58.8% of the 85 isolates analyzed harboured dsRNAs of viral characteristics. High heterogeneity was observed in the dsRNA profiles detected among strains, which indicated a high diversity of mycoviruses among *Beauveria bassiana* strains. In fact, a study of incidence of mycoviruses among *B. bassiana* isolates from a single holm oak revealed that the diversity of mycoviruses found in a reduced area (0.64 dsRNA profiles/infected isolate) was similar to that obtained among *B. bassiana* isolates collected throughout soils of Spain and Portugal (0.66 dsRNA profiles/infected isolate). Surprisingly, only two different dsRNA profiles were obtained among ten endophytic *B. bassiana* isolates infected by viruses, and collected at different locations in Spain. Nonetheless, the diversity of mycoviruses among *B. bassiana* isolates could be higher than that estimated from comparisons of dsRNA profiles. A hybridization using a DNA probe complementary to one of the detected mycoviruses showed that equal dsRNAs profiles did not always correspond to the same mycoviral infection. The complete genome of a new member of the *Victorivirus* genus was also obtained in the present work, *Beauveria bassiana* RNA virus 1 (BbRV1).

Introduction

Viruses have been detected in many species of fungi covering all four phyla of the true fungi: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota. In general, mycoviruses are very persistent, efficiently transmitted vertically to spores, and unlike animal or plant viruses, they normally infect their hosts asymptomatically (Romo *et al.,* 2007; Ghabrial and Suzuki, 2009; Herrero *et al.,* 2009). The symptomless phenotype of many mycovirus infections could be explained by the ancient infection hypothesis, reflecting a long period of coevolution in which reciprocal influences between the fungal host and mycoviruses would have evolved to a non virulent state of the virus, resulting in a symptomless virus-fungus association (Pearson *et al*., 2009). According to that, viruses could be even beneficial to their hosts under some conditions, and a mutualistic mycovirus which improves the thermal tolerance of a plant-fungal endophyte association could be an example of this situation (Marquez *et al.,* 2007). Nonetheless, all mycovirus associations are not consistent with this hypothesis, and a few mycoviruses are pathogenic to their hosts, causing hypovirulence in plant pathogens, or disease in mushrooms (Ghabrial and Suzuki, 2009; Nuss, 2005; Romaine and Goodin, 2002).

The presence of fungal viruses has been commonly diagnosed by the presence of double-stranded RNAs (dsRNAs), because most known mycoviruses have dsRNA genomes or single-stranded RNA (ssRNA) genomes producing dsRNA replicative intermediates (Morris and Dodds, 1979). The type of dsRNA elements observed in fungal isolates can be quite diverse, even in the same fungal species (Pearson *et al.,* 2009). This diversity can be noticed in terms of the number and size of the dsRNA elements detected in a fungal strain, and several dsRNAs of different sizes infecting the same fungus could indicate multipartite viral genomes, mixed infections, or even defective products of virus replication (Ghabrial and Suzuki 2008). Therefore, because of characteristics like the persistence or the efficient transmission to spores of mycoviruses, the polymorphic dsRNA profiles detected in fungi have been proposed as markers for distinguishing isolates of different origin within a species (Gillings *et al.,* 1993; Howitt *et al.,* 1995; Tooley *et al.,* 1989). These dsRNA profiles are sometimes associated to a geographical structure in some fungal species (Park *et al.,* 2006b; Voth *et al.,* 2006), but often they are not (Rong *et al.,* 2001; Tsai *et al.,* 2004).

The soil-inhabiting entomopathogenic fungus *Beauveria bassiana* is a natural enemy of a wide range of insects and arachnids, and has a cosmopolitan distribution

(Meyling and Eilenberg, 2007). Formulations of *B. bassiana* and other entomopathogenic taxa like *Metarhizium* or *Lecanicillium* are commercialized for the biological control of some agricultural pests (Mahmoud *et al.,* 2009). *B. bassiana* is one of the most widespread entomopathogenic fungal species in Spanish soils. In Spain and Portugal this fungus has preference for non-agricultural and clay soils, and by southern latitudes (Quesada-Moraga *et al.,* 2007). In addition to insects and other invertebrates *B. bassiana* is able to infect plants; the fungus has been recovered as an endophyte from several plant species (Vega *et al.,* 2008; Sánchez Márquez *et al.,* 2011). This endophytic capability has been demonstrated by artificial inoculation in different plant species of agricultural importance (Quesada-Moraga *et al.,* 2006a; Vega *et al.,* 2008). Therefore, according to the endophytic and entomopathogenic nature of *B. bassiana,* it has been proposed as a potential biocontroller against diverse insect plant pests. In addition, the fungus is known to produce an array of bioactive metabolites that limit the growth of some fungal plant pathogens, and it has been suggested that its endophytic colonization may induce systemic resistance against the pathogenic bacteria *Xanthomonas axonopodis* pathovar *malvacearum* in cotton (Ownley *et al.,* 2010). Because of this, it has also been considered as a potential biocontrol agent against plant pathogens.

B. bassiana and other hypocrealean entomopathogens like *Tolypocladium, Metarhizium, Paecilomyces,* or *Lecanicillium* spp. have been reported to harbour dsRNA elements showing polymorphic profiles (Melzer and Bidochka, 1998; Herrero *et al.,* 2009; Inglis and Valadares-Inglis, 1997). In *B. bassiana* isolates obtained from insects or plants in Brasil, North America, and Spain, dsRNA elements were detected, indicating virus infections in 16.7 to 66.7 % of the isolates analyzed. These dsRNA elements had sizes from 0.7 to 6 kbp, and the number of elements harboured per fungal isolate ranged from 1 to 5 (Melzer and Bidochka, 1998; Castrillo *et al.,* 2004; Dalzoto *et al.,* 2006; Herrero *et al.,* 2009). However, no sequences of these dsRNA elements are available at this date.

The main objective of the present work was to estimate the incidence, variability and distribution of dsRNA elements in soil and endophytic isolates of *B. bassiana* obtained in cultivated and natural areas of Spain and Portugal. The molecular characterization of some dsRNAs was an additional objective.

Materials and Methods

Fungal isolates

Eighty five isolates of *B. bassiana* were used for a survey of the incidence of mycoviruses in this species. Fifty four strains collected from soils in different habitats in cultivated and natural areas of the Iberian Peninsula and the Canary and Balearic Islands came from the collection of the Entomology Laboratory of the School of Agricultural and Forest Sciences and Resources (AFSR) from the University of Cordoba (Spain) (Table 1) (Quesada Moraga *et al.,* 2007). To study the local distribution of fungal viruses, sixteen isolates were collected at the same geographical point from the soil, phylloplane, and adventitious plants of a single holm oak (*Quercus ilex*), in Castilblanco de los Arroyos (Sevilla) (Table 2). Fifteen additional strains were isolated as endophytes from different grasses in natural habitats of Spain (Table 3). The viral infections harboured by these endophytic isolates were reported in a study of micoviruses in non-systemic endophytes of grasses (Herrero *et al.,* 2009).

Analysis of the presence of double-stranded RNA

The presence of dsRNA molecules of sizes ranging from 1 to 12 kbp was used as an indicator of virus infection in fungal isolates. This type of nucleic acid can represent the genome of dsRNA mycoviruses, as well as replicative forms of viruses with ssRNA genomes (Morris and Dodds, 1979). However, not all RNA viruses can be detected by dsRNA isolation (de Blas *et al*., 1996), and DNA viruses, recently discovered in fungi (Yu *et al*., 2010), would not be detected with this technique.

To detect the presence of dsRNA, each fungal isolate was cultured for three weeks over cellophane disks layered on top of potato dextrose agar (PDA) in Petri plates. Approximately 1.5 grams of fresh mycelium were harvested, ground with liquid nitrogen, and dsRNA was extracted by CF-11 cellulose chromatography (Morris and Dodds, 1979). To eliminate contaminating DNA, the purified dsRNA was treated with five units of DNase I for 30 minutes at 37 ºC, and extracted with one volume of phenol:chloroform (1:1). Contaminating ssRNA was removed by treatment with one unit of S1 nuclease at 37 ºC for 15 minutes, and extracted in the same way. DsRNA extracts were subjected to gel electrophoresis, and visualized after staining with ethidium bromide. All dsRNA extractions were independently repeated three times.

Table 1. *B. bassiana* isolates collected from soils of Spain and Portugal, and analyzed for the presence of dsRNA elements.

ISOLATE	LOCATION	HABITAT	ISOLATE	LOCATION	HABITAT
Bb 01/145	Sevilla	Olive grove	Bb 01/22	Córdoba	Scrubland
Bb 01/110	Sevilla	Oak grove	Bb 01/25	Córdoba	Olive grove
Bb 01/105	Sevilla	Cotton field	Bb 01/27	Córdoba	Wheat field
Bb 01/112	Sevilla	Wheat field	Bb 01/39	Málaga	Almond grove
Bb 01/103	Sevilla	Woodland	Bb 04/06	Córdoba	Cork oak grove
Bb 01/125	Cádiz	Fallow land	Bb 04/08	Córdoba	Hazel grove
Bb 01/33	Cádiz	Olive grove	Bb 00/08	Badajoz	Grassland
Bb 01/130	Cádiz	Pine forest	Bb 04/02	Cantabria	Source Ebro River
Bb 01/132	Cádiz	Cotton field	Bb 04/03	Cantabria	Grassland
Bb 01/15	Almería	Desert	Bb 04/05	Álava	Leek field
Bb 01/75	Almería	Beach	Bb 04/09	Madrid	Grassland
Bb 00/16	Almería	Scrubland	Bb 09/03	Ciudad Real	Eucalyptus forest
Bb 01/164	Huelva	Pine forest	Bb 09/04	Ciudad Real	Oak grove
Bb 01/168	Huelva	Scrubland	Bb 09/06	Ciudad Real	Eucalyptus forest
Bb 01/171	Huelva	Cotton field	Bb 09/07	Ciudad Real	Oak grove
Bb 01/19	Granada	Wheat field	Bb 09/08	Ciudad Real	Wild olive grove
Bb 01/64	Granada	Woodland	Bb 09/09	Ciudad Real	Olive grove
Bb 01/73	Granada	Scrubland	Bb 04/10	Gerona	Olive grove
Bb 07/08	Granada	Olive grove	Bb 06/01	Ibiza	Pine forest
Bb 01/34	Málaga	Olive grove	Bb 06/02	Fuerteventura	Fallow land
Bb 01/35	Málaga	Scrubland	Bb 06/03	Fuerteventura	Fallow land
Bb 01/36	Málaga	Meadow	Bb 07/15	Lugo	Fallow land
Bb 00/10	Jaén	Olive grove	Bb 08/08	Portugal	Olive grove
Bb 00/11	Jaén	Scrubland	Bb 08/09	Portugal	Olive grove
Bb 00/13	Jaén	Woodland	Bb 01/87	Portugal	Pine forest
Bb 01/43	Jaén	Olive grove	Bb 01/88	Portugal	Sunflower field
Bb 01/07	Córdoba	Meadow	Bb 01/89	Portugal	Unknown

The size of the different dsRNA elements was estimated in relation to DNA size standards (1 kb DNA ladder, Promega). All dsRNA bands, identified by their size, were listed in a matrix of all the strains analyzed. In this matrix the presence of a dsRNA element of a particular size in a fungal strain was indicated by a 1, and its absence by a 0. In order to cluster similar dsRNA profiles harboured by *B. bassiana* isolates, a dendrogram was built by the unweighted pair group method with arithmetic mean (UPGMA), based on the matrix of presence and absence of dsRNA bands in the isolates. For the construction of dendrograms the Statistica 5.0 software package was used (StatSoft, USA).

Table 2. Isolates of *B. bassiana* collected from a single holm oak (*Quercus ilex*) in Castilblanco de los Arroyos (Seville), and analyzed for the presence of dsRNA elements.

ISOLATE	HABITAT	ORIENTATION
B27	Phylloplane	West
B157	Phylloplane	West
B167	Phylloplane	West
B114	Adventitious plants	West
B124	Adventitious plants	West
B139	Phylloplane	East
B70	Phylloplane	East
B122	Phylloplane	South
B123	Phylloplane	South
B112	Phylloplane	North
B136	Phylloplane	North
B138	Phylloplane	North
Bs7	Soil	West
Bs20	Soil	East
Bs1	Soil	South
Bs5	Soil	North

Table 3. Endophytic *B. bassiana* strains collected from different species of grasses and analyzed for the presence of dsRNA elements.

ISOLATE	LOCATION	HOST
E 183	Salamanca	*Dactylis glomerata*
E 1764	Salamanca	*Dactylis glomerata*
E 2720	La Coruña	*Elymus farctus*
E 2773	La Coruña	*Ammophila arenaria*
E 2854	La Coruña	*Ammophila arenaria*
E 2857	La Coruña	*Elymus farctus*
E 3079	La Coruña	*Elymus farctus*
E 3080	La Coruña	*Elymus farctus*
E 3111	La Coruña	*Elymus farctus*
E 3154	Cáceres	*Holcus lanatus*
E 3155	Cáceres	*Holcus lanatus*
E 3158	Cáceres	*Holcus lanatus*
E 1923	La Coruña	*Ammophila arenaria*
E 2175	Cáceres	*Dactylis glomerata*
E 2980	La Coruña	*Elymus farctus*

cDNA synthesis

Strain Bb 06/02, harbouring a single dsRNA element of 5.8 kbp, was cultured for three weeks over cellophane disks layered on top of PDA Petri plates. The dsRNA was purified as explained before, and approximately 0.6 µg dissolved in water were used for cDNA synthesis. The RLM-RACE procedure described by Coutts and Livieratos (2003) was adapted in the present work for the construction of a cDNA library (Tuomivirta and Hantula, 2003). The cDNA products obtained were cloned in T-A vectors (Invitrogen). *Escherichia coli* strain DH5α was transformed and screened to select transformants containing inserts, which were sequenced. Gaps in the assembled sequences, which were not covered by clones derived from the cDNA library, were filled by reverse transcription and PCR primed by oligonucleotides designed according to sequences flanking the gaps. The ends of the molecule were cloned and confirmed using again the RLM-RACE method described by Coutts and Livieratos (2003) in three independent experiments.

Norhern blotting experiments

Northern blot hybridization was done in order to confirm if the 5.8 kbp dsRNA elements found in thirteen different strains (Bb 06/2, Bb 06/03, Bb 01/125, Bb 01/132, Bb 01/39, Bb 01/73, Bs1, Bb 09/03, Bb 09/08, Bb 01/75, Bb 07/08, Bb 09/04 and Bb 01/07) were sequence-homologous. DsRNA extracts from these isolates were electrophoresed in agarose gels, denatured, and transferred to nylon membranes (Zabalgogeazcoa *et al.*, 1998). Hybridization and detection was done using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). A clone from the 5' untraslated region (5'UTR) of the genome of *Beauveria bassiana* RNA virus 1 (BbRV1), which is a non conserved region in the genome of the *Totiviridae*, was used as a specific probe.

Sequence and phylogenetic analyses

Sequence similarity searches in the EMBL virus sequence database were conducted using the FASTA program (Pearson, 1990) (Table 4). For phylogenetic analyses sequence alignments were performed using the ClustalX program (Thompson *et al.*, 1997) and MEGA3 software (Kumar *et al.*, 2004). Genetic distances among amino acid sequences were calculated with the Poisson correction model. Phylogenetic trees were made using the neighbour-joining method, and bootstrap test values were based on 1000 replications.

Table 4. EMBL nucleotide database accession numbers of members of the family *Totiviridae* used for phylogenetic analysis.

Virus	Short name	Accession number
Armigeres subalbatus totivirus	AsTV	EU715328
Beauveria bassiana RNA virus 1	BbRV1	unpublished
Botryotinia fuckeliana totivirus 1	BfV1	CAM33265.1
Chalara elegans RNA virus 1	CeRV1	AY561500
Coniothyrium minitans RNA virus	CmRV	AAO14999.1
Drosophila melanogaster totivirus	DTV	GQ342961
Epichloë festucae vírus 1	EfV 1	CAK02788.1
Giardia lamblia virus	GLV	L13218
Gremmeniella abietina RNA virus-L1	GaRV-L1	AF337175
Gremmeniella abietina RNA virus-L2	GaRV-L2	AY615210
Helicobasidium mompa totivirus 1-17	HmV-17	BAC81754.1
Helminthosporium victoriae virus 190S	Hv-190SV	AAB94791.2
Infectious myonecrosis virus	IMNV	EF061744
Leishmania RNA virus 1-1	LRV 1-1	M92355
Leishmania RNA virus 2-1	LRV 2-1	U32108
Leishmania RNA virus 1-4	LRV 1-4	U01899
Magnaporthe oryzae virus 2	MoV-2	BAF98178.1
Omono River virus-AK4	OMRV-AK4	AB555544
Omono River virus-Y61	OMRV-Y61	AB555545
Saccharomyces cerevisiae virus LA	ScV L-A	J04692
Saccharomyces cerevisiae virus L-BC	ScV L-BC	U01060
Sphaeropsis sapinea RNA virus 1	SsRV 1	AAD11601.1
Sphaeropsis sapinea RNA virus 2	SsRV 2	AAD11603.1
Tolypocladium cylindrosporum virus 1	TcV 1	FR750562
Trichomonas vaginalis virus 2	TVV	AF127178
Ustilago maydis virus H1	UmV-H1	U01059

Transmission of dsRNA elements to asexual spores

Strain B123, which harboured ten different dsRNA elements, was used to study the efficiency of transmission of its dsRNA elements to asexual spores. This was done by analyzing the dsRNAs present in monosporic isolates derived from single conidia produced by strain B123. To obtain monosporic isolates, conidia were washed from PDA cultures of strain B123 with water containing 0.001% Tween 80 and a glass rod. The conidial suspension was centrifuged for 5 minutes at 800 x g. The pellet was resuspended in 200 µl of water, and different dilutions from this suspension were made and added to water agar plates. Single germinating conidia were observed under the microscope, collected with a needle, and transferred to PDA plates to obtain the monosporic isolates. To check the presence of dsRNA elements in the different monosporic cultures, dsRNA was purified and visualized as explained before.

Results

Incidence and patterns of dsRNA elements

Eighty five *B. bassiana* isolates were analyzed to study the incidence of mycoviruses among isolates collected in different habitats (soil, phylloplane of holm oak and associated adventitious plants, and internal tissues of grass leaves) in Spain and Portugal (Tables 1-3). Fifty of these isolates (58.8%) harboured dsRNA elements.

Mycovirus incidence was also analyzed separately according to the habitat and extent of the area from which the strains were isolated. Twenty nine out of fifty four (53.7%) strains collected from soils of natural and cultivated areas throughout Spain and Portugal harboured dsRNA elements (Figs. 1-2). The mycovirus incidence found in this group was the lowest, while that found among *B. bassiana* isolates sampled in the same geographical point from the soil, phylloplane and adventitious plants associated to a single holm oak (*Quercus ilex*) was the highest, 68.8% of the sixteen analyzed strains harboured dsRNA elements (Figs. 3-4). Two thirds (66.7%) of the fifteen endophytic isolates obtained from grasses were infected by viruses (Fig. 5). The differences found in the percentages of incidence in the three analyses were not large. In fact, the dsRNA incidences among endophytic isolates and those collected in the same sampling point were very similar.

Fig. 1. Electrophoretic banding patterns of dsRNA elements isolated from different strains of *B. bassiana.* Letters on the top indicate different isolates: A=Bb 01/39; B=Bb 01/75; C=Bb 01/33; D=Bb 01/132; E=Bb 06/02; F=Bb 06/03; G=Bb 09/03; H=Bb 09/08; I=Bb 00/08; J=Bb 00/11; K=Bb 01/15; L=Bb 01/35; M=Bb 01/110; N=Bb 09/07; O=Bb 01/88; P=Bb 08/09; Q=Bb 09/09; R=Bb 01/103. Lane MW contains a size marker and numbers on the left indicate kbp.

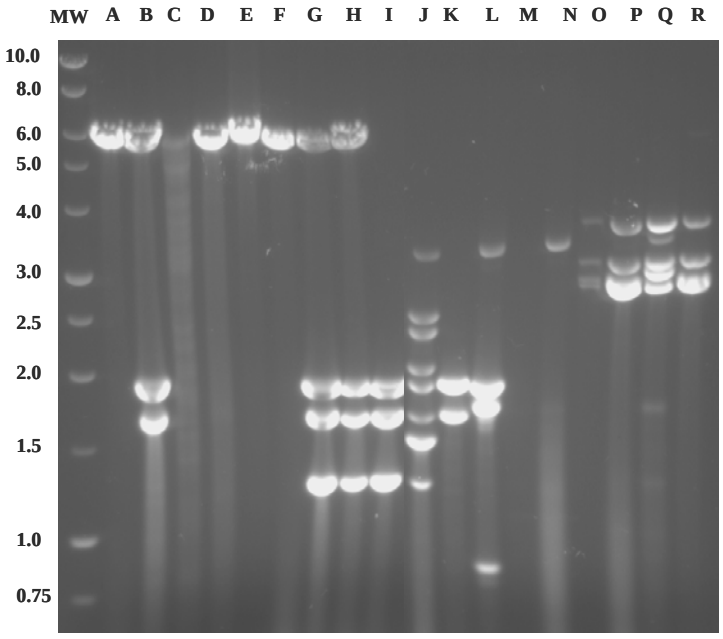


Fig. 2. Dendrogram constructed using the UPGMA method with euclidean distances showing the types of dsRNA infections observed in *B. bassiana* strains isolated from natural and cultivated soils of Spain and Portugal (Table 1). Numbers on the right indicate the sizes of the dsRNA elements harboured by the strains. Locations of isolation of the cultures are also showed in the diagram. Equal sets of dsRNA elements carried repeatedly by different *B. bassiana* isolates are placed inside the same colour box.

The heterogeneity of dsRNA profiles observed in the different *B. bassiana* strains also differed among and within the three subgroups analyzed. Some infected soil isolates contained only one element, while others had as many as eleven. The sizes of these dsRNA elements ranged from 0.8 to 5.8 kbp (Fig. 1). In order to group soil isolates harbouring similar dsRNA profiles, a tree diagram based in a similarity matrix of presence-absence of the different dsRNA elements observed in the survey was constructed (Fig. 2). Nineteen different dsRNA profiles were observed among soil strains, and while some isolates shared similar dsRNA profiles, others showed unique band patterns. A 5.8 kbp dsRNA was the most widespread element; it was present in twelve isolates (Fig. 1-2). This 5.8 kbp dsRNA was observed alone in some strains, and with other dsRNA elements in others. Therefore, the existence of mixed infections could be deduced from these combinations of dsRNA elements (Figs. 1-2).

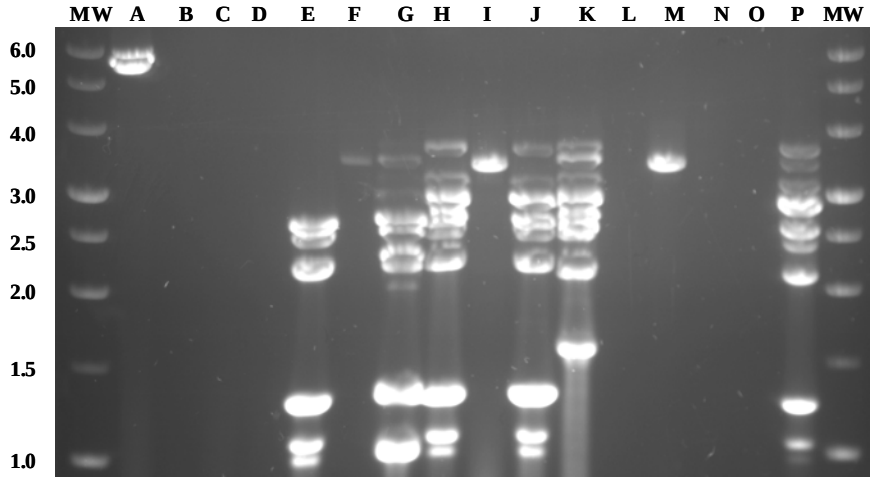
In addition, several dsRNA elements were observed always together in different strains: a set of two dsRNAs of 1.8 and 1.6 kbp occurred in seven soil isolates; another set constituted by 2.7, 2.4 and 2.1 kbp dsRNAs was present in six soil isolates; and a third set of 3.5, 3.2 and 3.1 kbp dsRNAs was found in three of the soil isolates (Fig. 2). Each of these sets of dsRNA elements could represent genomes of mycoviruses belonging to families with multipartite genomes like *Chrysoviridae* or *Partitiviridae* (Ghabrial *et al.,* 2005a,b).

In general, no concordance between similar dsRNA profiles and particular locations or habitats was found. For example, the 5.8 kbp dsRNA was found in isolates collected in different habitats in south and central Spain, and in the Canary Islands.

The reproduction and dissemination of *B. bassiana* is based mainly in the production of asexual spores. Transmission of dsRNA elements to asexual spores occur with frequencies close to 100% in some ascomycetes (Park *et al.,* 2006b; Romo *et al.,* 2007). According to this, in a reduced area *B. bassiana* clonal isolates harbouring similar dsRNA infections could be more easily found than in a large area. To check this hypothesis, a screening for the presence of dsRNA elements among *Beauveria* strains sampled in the same geographical point was made. Seven different dsRNA profiles were found among these isolates, but those observed in four strains (Bs27, B112, B114 and B124) were quite similar, in fact, these profiles had in common from six to ten dsRNA elements (Figs. 3-4). Three different sets of dsRNA elements were repeated in six isolates, and they could constitute genomes from multipartite viruses (Fig. 4). One of these sets,

consisting of three elements of 2.7, 2.4 and 2.2 kbp, was shared by six of the sixteen analyzed isolates, this profile was almost coincident with one of the profiles found among soil strains (2.7, 2.4 and 2.1 kbp); another set was composed of 1.3, 1.1 and 1.0 kbp elements, and was present in five isolates; and the last set consisted of four elements of 3.8, 3.6, 3.4 and 3.2 kbp, and was shared by other five isolates. The profile composed by the combination of these three sets of dsRNA elements was observed in B114, B123 and B167. On the other hand, the dsRNA element of 5.8 kbp, similar in size to the majority element obtained in the survey developed among soil strains, was unique to the Bs1 isolate. A 3.2 kbp element was the only harboured by B139. Strains B70, B122 and B138 harboured the same single dsRNA element of 3.6 kbp.

Fig. 3. Electrophoretic banding patterns of dsRNA elements isolated from different strains of *B. bassiana* sampled in the same geographical point (Castilblanco de los Arroyos - Seville). Letters on the top indicate different isolates: A=Bs1; B=Bs5; C=Bs7; D=Bs20; E=B27; F=B70; G=B112; H=B114; I=B122; J=B123; K=B124; L=B136; M=B138; N=B139; O=B157; P=B167. Lanes marked as MW contains size marker; numbers on the left indicate kbp.



*DsRNA element of 3.2 kbp harboured by B139 is not observable in this photograph due to its low concentration and the large electrophoretic time of run.

The number of different dsRNA profiles per infected strain collected in the same sampling point (0.64) was very similar to that observed with soil isolates (0.66). For that reason, in contrast with the expressed hypothesis above, *B. bassiana* clonal isolates harbouring similar dsRNA infections were not more common in a reduced area than in a large area. Surprisingly, the dsRNA patterns found among endophytic *B. bassiana* isolates were not as variable as those from soils or the same sampling point, only two different dsRNA profiles were found among them (Fig. 5). Actually, the endophytic *Beaveria*

strains collected from different locations of western Spain harboured 0.20 different dsRNA profiles per infected strain.

Fig. 4. Tree diagram constructed using the UPGMA method with euclidean distances showing the types of dsRNA infections observed in *B. bassiana* strains isolated from the soil, the phylloplane of a single holm oak tree, and adventitious plants under its canopy. Numbers on the right indicate the sizes of the dsRNA elements carried by the strains. Detailed locations of isolation of the cultures are also showed in the diagram: S=South, N=North, E=East, W=West. Equal sets of dsRNA elements harboured repeatedly by different *B. bassiana* isolates are placed inside the same colour box.

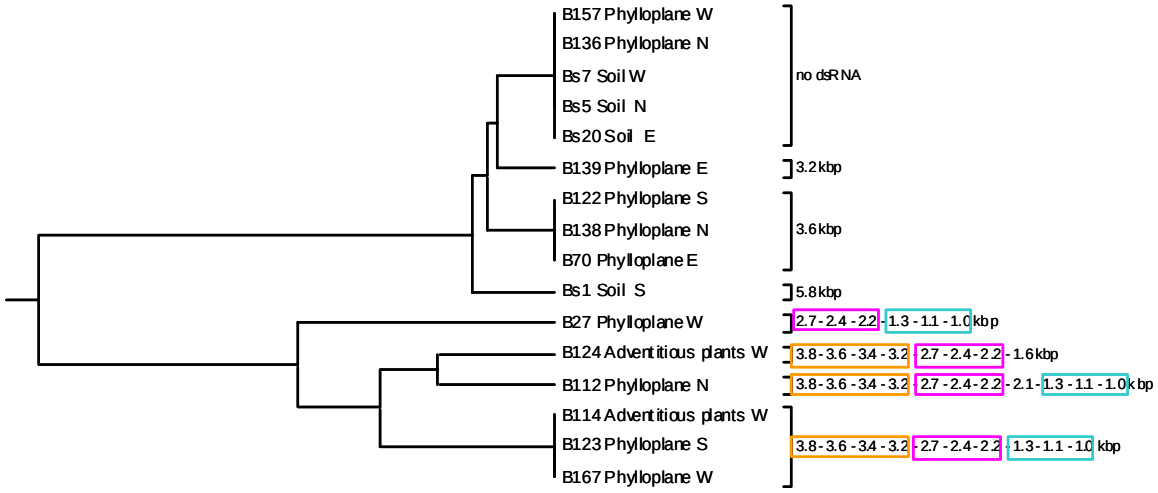
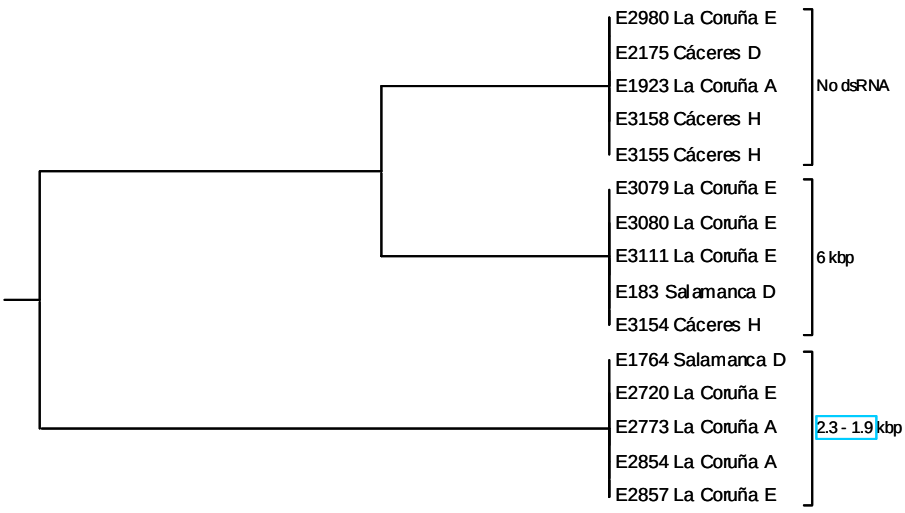


Fig. 5. Tree diagram constructed using UPGMA method with euclidean distances showing the dsRNA infections found in each analyzed endophytic *B. bassiana* strains isolated from different grass species of natural grasslands of western Spain. Numbers on the right indicate the sizes of the dsRNA elements carried by the strains. Locations and host of isolation of the cultures are also showed in the diagram. Hosts from which endophytes were obtained were: E= *Elymus farctus*, D= *Dactylis glomerata*, A= *Ammophila arenaria*, *H= Holcus lanatus*. Equal sets of dsRNA elements harboured repeatedly by different *B. bassiana* isolates are placed inside the same colour box.



Transmission of dsRNA elements to asexual spores

The study of transmission of dsRNA elements to mitotic spores in strain B123 revealed that the 40 monosporic isolates obtained from this strain maintained the original viral infection composed by 10 dsRNA elements.

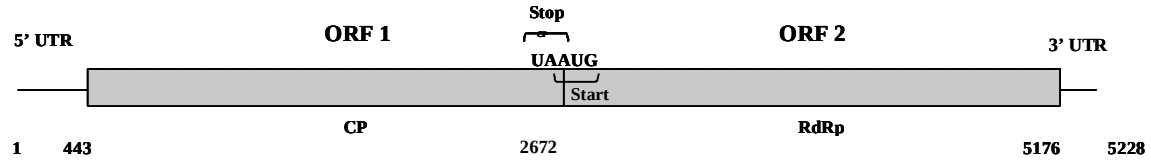
Nucleotide sequence and genome organization of the viral genome

The 5.8 kbp dsRNA which was the most abundant element in the survey was sequenced. Forty two different clones from a cDNA library were sequenced, and four contigs were obtained from their assembly. The gaps between the four contigs were resolved using specific primers flanking the gaps. These experiments were repeated three times. Six identical clones of the 5' end and four identical clones of the 3' end from two independent RLM-RACE experiments of each terminus were sequenced.

The complete sequence of the dsRNA element harboured by strain Bb 06/02 is 5228 bp in length, and has a GC content of 55% (Fig. 6). It has two continuous open reading frames (ORFs): ORF 1 has a length of 2229 bp and encodes a 742 amino acid protein (78.41 kDa); ORF 2 is 2505 bp long and encodes an 834 amino acid protein (91.15 kDa). These two ORFs are separated by a pentanucleotide, UAAUG, that constitute the stop codon of ORF1 and the start codon of ORF2, and they overlap by one nucleotide. Other ORFs longer than 528 nt were not found in any strand. The 5' and 3' UTRs had 443 and 52 bp, respectively. The 5' UTR starts with a GAATA sequence similar to a GAAAA motif that was proposed to have an important role in RNA replication and transcription in *Ustilago maydis* Virus (UmV), and *Saccharomyces cerevisiae* viruses -LA and L1 (ScV-LA, ScV-L1) (Fujimura and Wickner, 1988; Kang *et al.*, 2001).

The amino acid sequence deduced from ORF1 exhibits a high degree of identity to those of the capsid proteins (CP) of viruses of the family *Totiviridae*, particularly to that of *Helmintosporium victoriae* virus 190S (Hv-190SV; 56.6%). The C-terminus of this putative CP has an Ala/Gly/Pro-rich region, which is shared by viruses in the *Victorivirus* genus (Ghabrial and Nibert, 2009). The deduced amino acid sequence of ORF2 resembled those of RdRps of viruses of the family *Totiviridae*, particularly that of *Sphaeropsis sapinea* RNA virus 1 (SsRV-1; 45.3% identity). The eight conserved motifs of the sequences of RdRps of dsRNA viruses of simple eukaryotes (Bruenn, 1993) were found in the amino acid sequences deduced from the ORF 2.

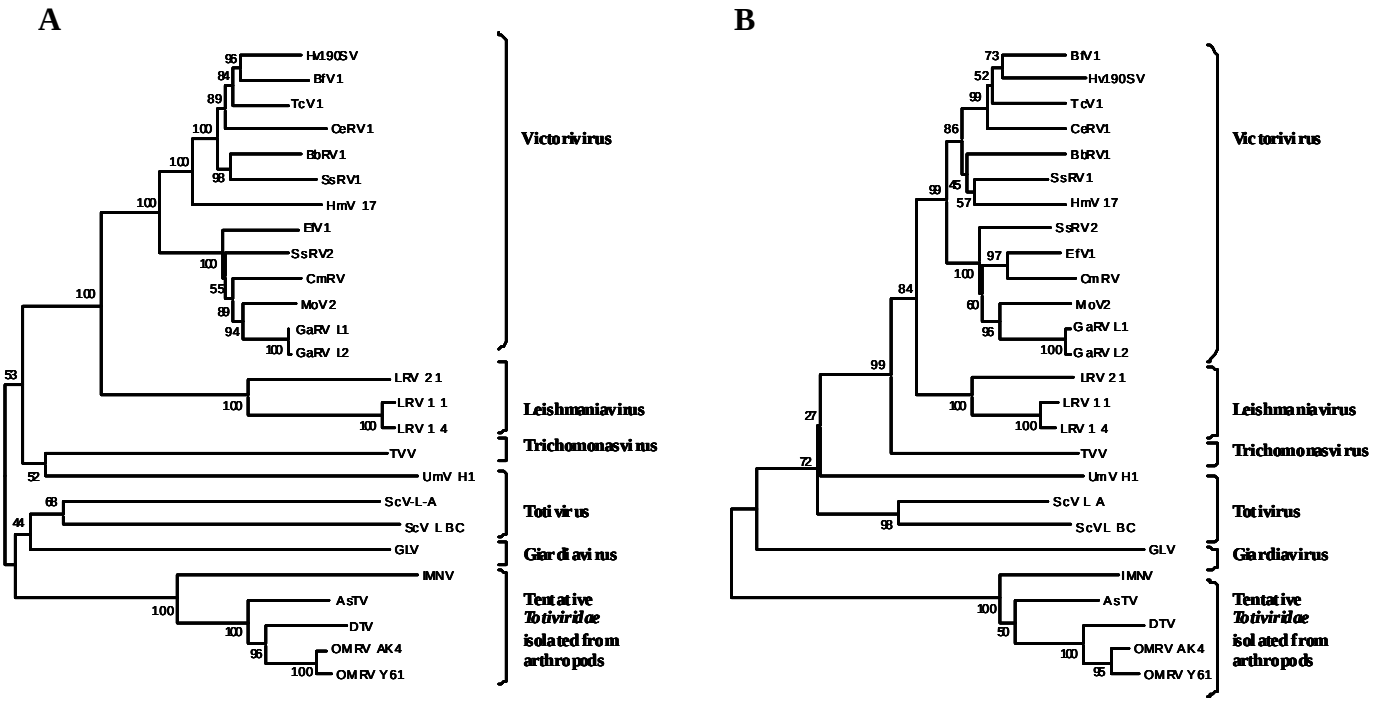
Fig. 6. Genome organization of *Beauveria bassiana* RNA Virus 1 (BbRV 1). The 5228 bp genome contains two ORFs; ORF1 encodes a putative CP and ORF2 a putative RdRp.



Phylogenetic analysis of Beauveria bassiana RNA virus 1

Phylogenetic analyses based on the complete amino acid sequence of the CP and RdRps of selected members of the *Totiviridae* family and those deduced from ORF1 and ORF2 of the 5.8 kbp dsRNA were done (Fig. 7A). These two phylogenetic trees revealed that the dsRNA infecting strain Bb 06/02 resembles most the genomes of mycoviruses included in a clade within the genus *Victorivirus*. This genus is composed by viruses which infect filamentous fungi (Ghabrial and Nibert, 2009). Therefore, these phylogenetic analyses, and other mentioned characteristics of the dsRNA infecting *B. bassiana* strain Bb 06/02, like having a non-segmented dsRNA genome of 4.6-6.7 kbp coding for a CP and an RdRp, having a Pro/Ala/Gly-rich region near the C-terminus of the CP, and a 5' and 3' UTRs with sizes similar to those from the genus *Victorivirus* (Wickner *et al.*, 2005; Ghabrial and Nibert, 2009), indicated that this dsRNA element represents the complete genome of a new member of this genus, *Beauveria bassiana* RNA virus 1 (BbRV1).

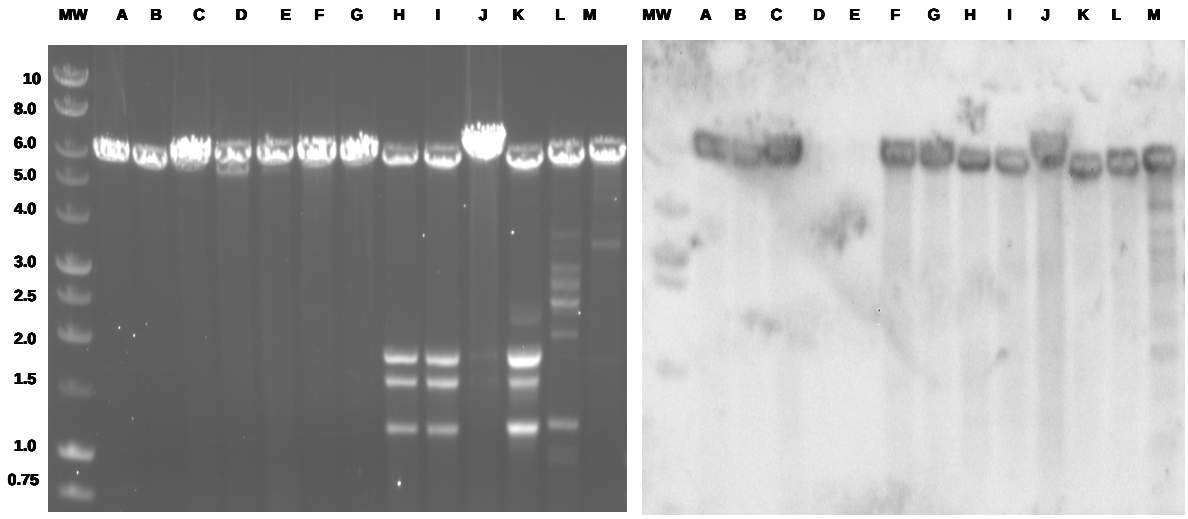
Fig. 7. Neighbor-joining phylogenetic trees of viruses of the family *Totiviridae* based on CP amino acid sequences (**A**) and on RdRp amino acid sequences (**B**). The unrooted phylogenetic trees based on the neighbour-joining method were created using MEGA software. Numbers at nodes represent bootstrap values as percentages estimated by 1000 replicates. The accession numbers of sequences used in the analyses are given in Table 4.



Northern blotting experiments

A Northern blot hybridization was made to check if *B. bassiana* isolates sharing the 5.8 kbp dsRNA profile are infected by the same mycovirus. A 543 nucleotide (nt) clone complementary to the 5' UTR of the genome of BbRV 1 was used as probe. This probe represents a non conserved area among members of the *Totiviridae*, and its specificity for BbRV1 is likely to be high. Only eleven of the thirteen isolates of *B. bassiana* harbouring the 5.8 dsRNA hybridized with the BbRV1 probe (Fig. 8). Therefore, although strains Bb 01/132 and Bb 01/39 harbour a single 5.8 kbp dsRNA element, they seem to be infected by a mycoviral species different to BbRV1.

Fig. 8. Northern blot experiment using as probe a clone belonging to BbRV 1. Letters on the top indicate different isolates: A=Bb 06/2; B=Bb 06/03; C=Bb 01/125; D=Bb 01/132; E=Bb 01/39; F=Bb 01/73; G=Bs1; H=Bb 09/03; I=Bb 09/08; J=Bb 01/75; K=Bb 07/08; L=Bb 09/04; M=Bb 01/07. Lane MW contains a size marker and numbers on the left indicate kbp.



Discussion

This study of incidence showed that mycoviruses are widespread among *B. bassiana* isolates collected from different habitats in Spain and Portugal, 58.8% of the 85 isolates analyzed harboured dsRNAs of viral characteristics. Mycovirus incidence was also analyzed separately according to the habitat or the extent of the area where isolates were collected. The incidence observed among soil isolates was the lowest (53.7%), while those obtained for isolates collected from soil, phylloplane or adventitious plants associated to a single holm oak (68.8%) or among endophytic isolates (66.7%) were very similar. These values are higher than the 16.7-23% incidences obtained in other surveys of viruses in *B. bassiana* carried out in Brasil or North America; however, in each of these studies less than eight isolates were analyzed (Melzer and Bidochka, 1998; Castrillo *et al*., 2004; Dalzoto *et al.*, 2006).

Mixed virus infections could be deduced from the dsRNA profiles observed; in several isolates the same dsRNA element or a set of dsRNAs were observed alone, but in other isolates the same element or elements were accompanied by other dsRNAs. For example, the 5.8 kbp dsRNA which corresponds to the genome of BbRV1 was found alone in some isolates, and together with other dsRNAs of viral characteristics in others (Figs. 1

and 8). Mixed mycovirus infections have been reported for other fungal species, including the entomopathogenic fungus *Tolypocladium cylindrosporum* (Ghabrial and Suzuki, 2008; Herrero *et al.,* 2011).

The large number of different dsRNA profiles obtained among all the *B. bassiana* strains analyzed suggests that there is an important diversity of mycoviruses associated to this entomopathogenic species. Although partial or complete sequences of all dsRNA elements would be necessary for their correct classification within a mycovirus family, the observed electrophoretic band patterns could be helpful for a hypothetical classification of *Beauveria* mycoviruses. Some sets of dsRNAs found repeteadly among isolates could correspond to the multipartite genome of a single virus. For instance, some dsRNA profiles could correspond to members of the families *Partitiviridae* (bipartite genomes of 1.4-2.2 kbp), *Chrysoviridae* (genomes composed by four segments of 2.4-3.6 kbp). One of the three patterns consisting of a single dsRNA band (5.8 kbp) was confirmed by sequencing to belong in most isolates to BbRV1, a member of the *Totiviridae* (non-segmented genomes of 4.6-6.7 kbp) (Figs. 2-4) (Ghabrial *et al.*, 2005a,b; Wickner *et al.,* 2005). Intermediates of replication of members of the *Barnaviridae* family could also be harboured by some isolates (genomes formed by a single ssRNA of ca. 4 kbp) (Fig. 2) (Revill, 2010). Other dsRNA elements observed did not show characteristics of known mycovirus families and could constitute members of new families, satellite RNAs, or defective derivates of replication (Yao *et al.,* 1995; Zhang and Nuss, 2008).

The three observed dsRNA sets observed among the isolates collected from the phylloplane and the adventitious plants associated to a single holm oak could constitute multipartite genomes of single viruses (Fig. 4). Alternatively, they could constitute the complete genome of a member of the *Mycoreoviridae* family, since these three sets of dsRNA elements were found together in four of the isolates (B112, B114, B123 and B167) and *Mycoreoviridae* members have genomes composed by 10 to 12 segments of 0.7-4.1 kbp (Hillman, 2010). In addition, rearrangements of individual genome segments, including extensions and/or delections of ORFs, have been described as common to members of the *Reoviridae* (Eusebio-Cope *et al.,* 2010), and for that reason, strains Bs27 and B124 could harbour the same mycoreovirus as B112, B114, B123 and B167, but with some rearrangements in their genomes. This result would decrease the diversity of mycoviruses found among *B. bassiana* strains from the same area. The dsRNA profile information would indicate that the virus diversity among soil isolates collected throughout

Spain and Portugal (0.66 dsRNA profiles/infected isolate) is higher to that observed around a single tree, if the mycorreovirus hypothesis would be true (0.36 dsRNA profiles/infected isolate). This result could support the hypothesis suggesting that in a reduced area, *B. bassiana* clonal isolates harbouring similar dsRNA infections could be more easily found than in a large area.

In addition to the large diversity indicated by dsRNA profiles, the Northern blot experiments with a BbRV1 probe revealed that the 5.8 kbp dsRNA band was not sequence-homologous to thirteen strains showing this dsRNA element (Fig. 8). Therefore, similar dsRNA profiles do not imply infections by the same mycoviruses, and a higher diversity of mycovirus species than that predicted by electrophoretic dsRNA profiles could occur among the *B. bassiana* isolates.

In contrast with the relatively high variation of dsRNA patterns found among *B. bassiana* isolates collected from soil (0.66 dsRNA profiles/ infected isolate) or from soil, phylloplane, and adventitious plants of a single holm oak (0.64 dsRNA profiles/ infected isolate), only 0.20 dsRNA profiles per isolate analyzed were found among the fifteen endophytic strains analyzed. Five isolates harboured a 6 kbp dsRNA element similar in size to BbRV1, which could be a member of the *Totiviridae*; other five strains harboured a set of a 2.3 and a 1.9 kbp dsRNAs, which could correspond to the bipartite genome of a partitivirus, and another five isolates were virus-free. No relationship between the dsRNA profile and the location or species of the grass host was found. The lower diversity of profiles found among these isolates could be associated with their endophytic habit. Some taxonomic studies indicate that *B. bassiana* might be a species complex that includes several cryptic species (Rehner and Buckley, 2005). According to that, endophytic *B. bassiana* isolates could represent a species different to those collected from other different habitats. If this situation occurred, then only certain mycoviruses could be associated to endophytic strains. Another hypothetical explanation could be that some mycoviruses might be more compatible with an endophytic lifestyle than others. With this condition, the endophytic environment could act as a filter against some viruses.

The results of the study of transmission of dsRNAs to asexual spores, showed a 100% transmission of the ten dsRNAs harboured by strain B123 to its conidial progeny. Similar rates of transmission of dsRNA elements to conidia have been observed in other ascomycetes, even when they harboured mixed virus infections (Chu *et al.*, 2004; Tuomivirta and Hantula, 2005; Romo *et al.*, 2007). Nevertheless, in *Tolypocladium*

cylindrosporum, another entomopathogenic species, different rates of transmission were found for each of the three mycoviruses that infect it (Herrero *et al.,* 2011). The same could occur among *B. bassiana* isolates, and the different combinations of similar dsRNA elements or sets of dsRNAs found among strains could be explained by different rates of transmission for each mycovirus. If transmission rates to conidia would differ among viruses in mixed infections, then dsRNA profiles would not be good markers for distinguishing between different strains of *B. bassiana*, since the dsRNA profiles detected in this species would be unstable.

In addition to conidia, dsRNA elements might also be transmitted among compatible strains of *B. bassiana* by hyphal anastomoses; however, the large number of vegetative compatibility groups existing in the species may limit this type of transmission (Castrillo *et al.,* 2004). Horizontal transfer of dsRNA has been reported between incompatible strains of the same species or between closely related species, this process seems to occur with very low frequency and it is not stable (Charlton and Cubeta, 2007; Melzer *et al.,* 2002). The existence of a large number of different dsRNA profiles observed in this study suggests that transmission by anastomoses between strains is not common. However, if in multiple infections the rate of transmission would differ among viruses, losses of individual viruses could occur, and this fact would explain also the existence of the large number of different dsRNA profiles observed in this study.

The 5.8 kbp dsRNA was the most common element detected in the survey. Its size, sequence, and genome organization, indicate that this dsRNA constitutes the complete genome of a new member of the *Victorivirus* genus (Family: *Totiviridae*), *Beauveria bassiana* RNA virus 1 (BbRV1). The 5228 kbp dsRNA genome of BbRV1 has characteristics of the *Totiviridae* family (Wickner *et al.,* 2005): contains two ORFs that overlap by one nucleotide (UAAUG), ORF1 encodes a CP and ORF2 an RdRp. The phylogenetic analysis grouped this virus within the *Victorivirus* genus, and as other members of this genus it has a Pro/Ala/Gly-rich region near the C-terminus of the CP, and 5' and 3' UTRs with sizes similar to those of other victoriviruses (Ghabrial and Nibert, 2009). For the expression of the RdRp, BbRV1 may follow the same strategy as other victoriviruses, a coupled termination-reinitiation mechanism (Ghabrial and Nibert, 2009). Another victorivirus infecting an entomopathogenic fungus has been recently described, *Tolypocladium cylindrosporum* virus 1 (TcV1) (Herrero and Zabalgogeazcoa, 2011). It is interesting that totiviruses have entomopathogenic fungi and insect hosts (Isawa *et al.,*

2010; Wu *et al.*, 2010). Could a host jump have occurred from the fungi to the insect or vice versa?

The strains harboring BbRV1 were obtained in diverse habitats and locations (Fig. 2); this virus was found in strains from the south and center of the Iberian Peninsula, and in the Canary Islands. The explanation of how isolates from distant locations harboured BbRV1 may be found in the population dynamics of *B. bassiana*. As other entomopathogenic fungi, *B. bassiana* is passively dispersed by the action of weather components like wind and rain, but it can be also dispersed by living infected hosts which may migrate long distances and die far from where they became infected (Meyling and Eilenberg, 2007). This way of dispersion could also explain why a clear geographical distribution of isolates harboring similar viral infections was not found in this analysis. The same was observed in a study of viruses in another entomopathogenic fungus, *Metarhizium anisioplae* (Melzer and Bidochka, 1998).

The effects that dsRNA infections produce in their hosts were not evaluated in the present work; further molecular characterization of all the dsRNA elements harboured by *B. bassiana* could help to study these possible effects. Such studies would also clarify our hypothetical taxonomical classifications based on dsRNA size and numbers.

3. DISCUSIÓN GENERAL

3.1. Incidencia y diversidad de micovirus en hongos endofíticos y entomopatógenos.

Infecciones por micovirus han sido descritas en miembros de los cuatro phyla de los hongos verdaderos: Chytridiomycota, Zygomycota, Ascomycota y Basidiomycota. De hecho, varios estudios de incidencia de micovirus en especies de hongos como *Monilinia fructicola*, *Chalara elegans*, *Botrytis cinerea*, *Aspergillus* spp., *Metarhizium anisopliae* ó *Ustilago maydis* muestran incidencias muy elevadas dentro de una especie, que pueden llegar incluso al 100% (Bottacin *et al.,* 1994; Howitt *et al.,* 1995; Melzer y Bidochka, 1998; Tsai *et al.,* 2004; Van Diepeningen *et al.,* 2006; Voth *et al.,* 2006). En cambio, parece haber algunas excepciones a esta ubicuidad, así, nunca se han encontrado virus en *Neurospora crassa*, y la causa parece residir en un mecanismo de defensa del hongo que se basa en procesos de silenciamiento por RNA (Pearson *et al.,* 2009). Ahora bien, aunque existe cierta evidencia de que el silenciamiento por RNA actúa como mecanismo de defensa en los hongos (Segers *et al.,* 2007), no parece ser muy eficiente contra los virus y se habría evolucionado a una situación en la que el parásito permanece causando el mínimo impacto en su hospedador. Este tipo de asociación virus-hongo recuerda bastante a las ya comentadas interacciones asintomáticas entre hongos endofíticos y plantas.

De acuerdo con este paralelismo endofito-micovirus y con el hecho de que los estudios de incidencia de micovirus que existían hasta el momento se centraban únicamente en una determinada especie de hongos, sin dar una idea de cuán comunes son los micovirus entre los hongos, en esta memoria se pretendió comprobar si también existía un paralelismo endofito-micovirus en cuanto a la ubicuidad, puesto que hasta la fecha no se conoce una sola especie vegetal sin una micobiota endofítica asociada (Arnold, 2007). De este modo, el estudio de la incidencia de micovirus dentro del grupo de los hongos endofíticos no sistémicos de gramíneas puso de manifiesto que los micovirus son comunes dentro de este grupo de hongos, encontrándose en cerca del 25% de las especies analizadas. Ahora bien, en este estudio el número de aislados analizado por especie varió bastante, desde un único aislado por especie hasta siete u once en el caso de *Beauveria bassiana* y *Torrubiella confragosa.* En vista de que en los casos en que se analizaron varios aislados de una misma especie de hongo las incidencias de micovirus obtenidas fueron más elevadas, el 67% de los aislados analizados de *B. bassiana* y el 46% de los aislados de *T. confragosa* resultaron infectados por micovirus, el 25% de incidencia de micovirus obtenido para el conjunto de especies de hongos endofíticos no sistémicos de

gramíneas podría ser una infraestimación. Así, si se aumentase el número de aislados analizados por especie, es muy probable que la incidencia de virus entre especies lo hiciera también.

Es preciso resaltar que entre los endofítos no sistémicos de gramíneas analizados en el estudio de incidencia se encontraron infecciones por virus en tres especies que a su vez son entomopatógenas *B. bassiana*, *T. confragosa* y *Tolypocladium cylindrosporum*. Existen otros trabajos que describen la presencia de micovirus en otros hongos entomopatógenos como *Metarhizium anisopliae*, *Paecilomyces* spp. y *B. bassiana* (Dalzoto *et al.,* 2006; Inglis y Valadares-Inglis, 1997; Melzer y Bidochka, 1998). Ahora bien, en este trabajo se describen por primera vez infecciones por micovirus en *T. confragosa* y *T. cylindrosporum.*

Los hongos entomopatógenos constituyen un grupo que en la actualidad despierta un gran interés como alternativa al uso de sustancias químicas en la lucha contra plagas de insectos herbívoros, garrapatas, o vectores de enfermedades vegetales, animales o humanas (Kaaya y Hassan, 2000; Scholte *et al.,* 2004; Quesada-Moraga *et al.,* 2006). Por otro lado, desde que se conoce su naturaleza endofítca, el interés hacia estos hongos se ha incrementado aún más de cara a su aplicación en agricultura (Ownley *et al.,* 2010; Vega *et al.,* 2008). Adicionalmente, hongos entomopatóngenos como *B. bassiana* o *T. cylindrosporum* presentan un atractivo añadido, y es que son productores de sustancias de interés médico como la ciclosporina, que es un inmunosupresor empleado en trasplantes de órganos para evitar rechazos (Aarnio y Agathos, 1989). Estas características de los hongos entomopatógenos hicieron que en esta tesis los estudios se centrasen, de entre todas las especies de hongos endofíticos analizados, en el estudio de micovirus asociados a hongos entomopatógenos. Así, se llevaron a cabo dos estudios de incidencia de micovirus en *T. cylindrosporum* y *B. bassiana*.

En el análisis de incidencia de micovirus en *T. cylindrosporum* se incluyeron tres aislados endofíticos y otras nueve cepas aisladas de suelos e insectos. Se detectaron virus en el 45.5 % de los aislados analizados. En el estudio de incidencia de micovirus en cepas de *B. bassiana* de áreas naturales y cultivadas de España y Portugal, se partió de un total de 85 aislados, incluyendo 15 aislados endofíticos. El resto de los aislados incluidos en este estudio procedían de otros tipos de hábitat que *B. bassiana* es capaz de colonizar: suelos, filoplano y superficie de plantas adventicias. En este trabajo se detectaron micovirus en el 62.5 % de los aislados de *B. bassiana*.

La diversidad de micovirus observada en los tres estudios de incidencia fue elevada. De los micovirus de los que no se obtuvo una secuencia parcial o completa, se pudo especular acerca de su clasificación basándose en el tamaño y número de los elementos de dsRNA observados. De este modo, miembros de las familias *Totiviridae*, *Chrysoviridae*, *Partitiviridae*, *Barnaviridae* o incluso *Mycoreoviridae*, podrían infectar a distintas especies de endofitos, así como a diferentes aislados de *T. cylindrosporum* y *B. bassiana*. No obstante, la diversidad de micovirus encontrada en estos estudios de incidencia podría ser mayor que la estimada según la diversidad de perfiles de dsRNA detectados. Esto se debe a que una hibridación realizada con una sonda de DNA complementaria a un área del genoma de uno de los virus secuenciados en esta tesis (BbRV1) demostró que patrones similares de dsRNA no son siempre indicativos de una infección por una misma especie de micovirus.

Las infecciones mixtas parecen ser comunes entre los hongos, de hecho, este tipo de infecciones han sido descritas en diversas especies de hongos e incluso en la especie endofítica *Epichloë festucae* (Ghabrial y Suzuki, 2008; Romo *et al.*, 2007). En esta tesis se detectó una infección triple en un aislado de *T. cylindrosporum,* que alberga a TcV1, TcV2 y TcV3. Este tipo de infecciones también se detectaron en *B. bassiana* y del mismo modo podrían ocurrir en muchos de los aislados analizados que contienen múltiples elementos de dsRNA. Por otra parte, los micovirus presentes en una infección mixta suelen tener tasas de transmisión a las esporas asexuales similares entre sí. Sin embargo, en esta tesis se describieron por primera vez tasas de transmisión diferentes para cada uno de los virus que infectaban a *T. cylindrosporum.*

Dentro de *B. bassiana* encontramos un fuerte contraste entre la diversidad de micovirus encontrada en las cepas aisladas de suelo, filoplano o superficie de planta adventicia, y la diversidad de micovirus en cepas aisladas como endofitos de gramíneas. Así, mientras que en las cepas de *B. bassiana* aisladas de suelo, filoplano o superficie de planta adventicia se encontraron hasta 26 perfiles de dsRNA diferentes, en las cepas endofíticas sólo se encontraron 2 perfiles. Esto sugiere que los aislados endofíticos podrían tal vez diferir de los obtenidos en otros hábitats. Por otro lado, no se encontró relación entre el tipo de infección, sustrato, hospedador o localidad de origen del aislado. Así, en aislados de *B. bassiana* procedentes de un mismo punto de muestreo se llegaron a encontrar hasta 7 distintos perfiles de dsRNA. Por el contrario, se encontró el mismo tipo de infección en varios aislados endofíticos procedentes de distintos puntos de la península

y de distintos hospedadores. De este modo, dada la poca variabilidad encontrada dentro de las infecciones por micovirus en aislados endofíticos de *B. bassiana*, y la elevada diversidad de perfiles de dsRNA encontrada en aislados procedentes de suelo, filoplano y planta adventicia, la capacidad endofítica de *B. bassiana* podría estar relacionada con determinadas infecciones virales. Otra explicación podría basarse en el hecho de que el taxón *B. bassiana* podría albergar especies crípticas (Rehner y Buckley, 2005), así, los aislados endofíticos podrían representar un taxón distinto al resto de aislados del estudio, de tal forma que sólo determinados micovirus serían característicos de las cepas endofíticas.

Por otro lado, el hecho de que en los aislados endofíticos analizados de *T. confragosa* se encontraran infecciones similares a aquellas encontradas en aislados endofíticos de *B. bassiana*, podría estar relacionado con el hecho de que ambos hongos pertenecen a la familia *Cordycipitaceae*, de forma que estos virus podrían haber estado presentes en un ancestro y llevarían ligados a estos taxones desde antes de que se produjera su especiación. No obstante, sería necesaria la secuenciación de estos virus para poder afirmar que perfiles semejantes de dsRNA se comprenden con las mismas especies de micovirus, lo cual no siempre ocurre, como así lo demostró la hibridación realizada usando como sonda un área específica del genoma de BbRV1.

3.2. TcV1 y BbRV1, dos nuevos victorivirus que infectan hongos entomopatógenos.

En esta tesis se describen por primera vez dos micovirus que infectan a hongos entomopatógenos, TcV1 infecta a *T. cylindrosporum* y BbRV1 a *B. bassiana.* Así, aunque se han detectado micovirus infectando otras especies entomopatógenas como *B. bassiana, M. anisopliae* o *Paecilomyces* spp. (Dalzoto *et al.*, 2006; Inglis y Valadares-Inglis, 1997; Melzer y Bidochka, 1998), ninguno de estos virus había sido secuenciado e identificado hasta el momento.

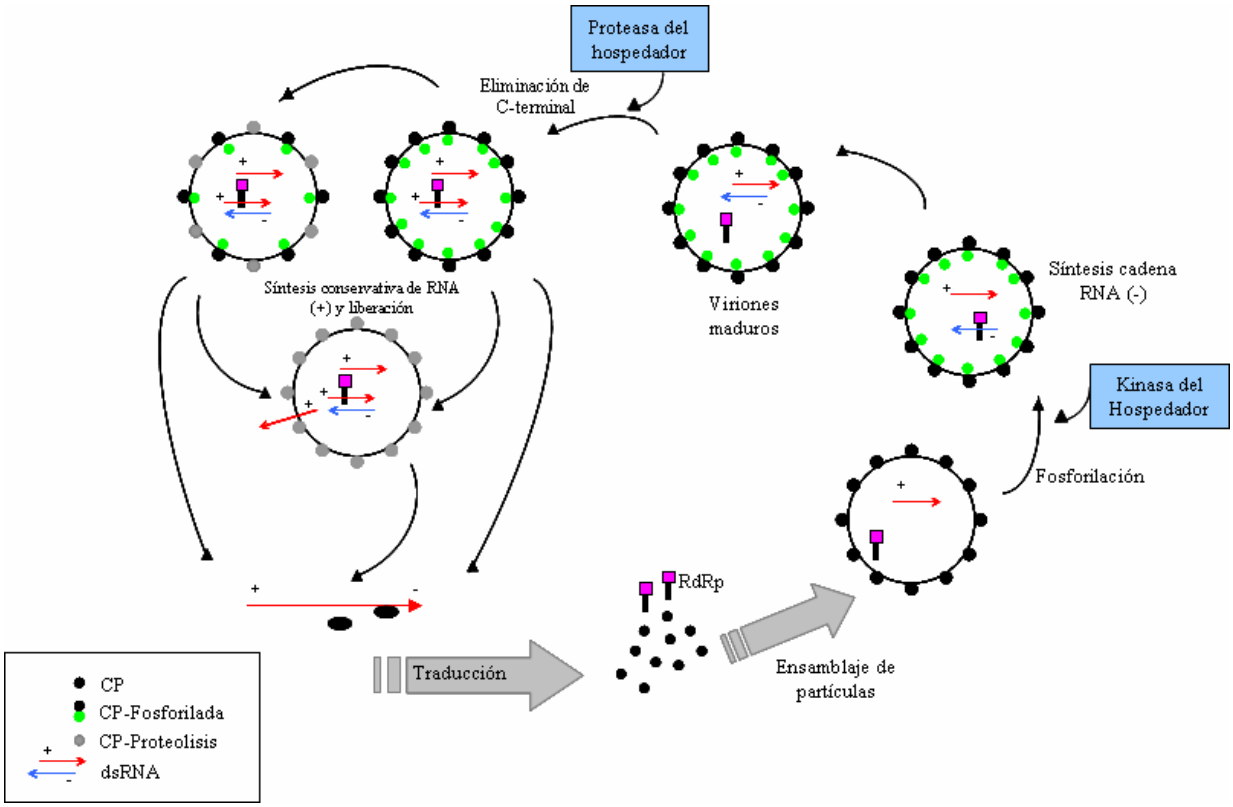
Estos dos nuevos virus tienen un genoma de dsRNA cuyas secuencias completas tienen 5196 bp en el caso de TcV1 y 5228 bp en el caso de BbRV1. Ambos presentan genomas con una organización característica de la familia *Totiviridae*, es decir, genomas no segmentados de dsRNA de entre 4.6 y 6.7 kbp que codifican una proteína de cápsida (CP) y una RNA polimerasa dependiente de RNA (RdRp) (Wickner *et al.*, 2005; Ghabrial,

2010). Como es típico de totivirus, los viriones de TcV1 tienen un diámetro de cerca de 50 nm y la proteína mayoritaria en un análisis de SDS-PAGE de partículas de virus purificadas resultó tener un tamaño aproximado de 79 kDa, lo esperado de la traducción del ORF 1 de este virus que codifica una proteína de cápsida. Por otro lado, estudios filogenéticos basados en el alineamiento de secuencias de aminoácidos de CP y RdRp de estos dos virus y otros virus pertenecientes a los diferentes géneros que componen la familia *Totiviridae*, revelaron que TcV1 y BbRV1 agrupan perfectamente dentro del género *Victorivirus*. De hecho, ambos virus comparten características propias de este género: infectan hongos filamentosos, poseen una región rica en Pro/Ala/Gly en la región cercana al C-terminal de la CP, y tienen UTRs con tamaños que se ajustan a los del resto del género, 61-574 nucleótidos (nt) para 5' UTR y 43-114 nt para 3' UTR (Ghabrial y Nibert, 2009). Además al igual que otros victorivirus, TcV1 y BbRV1 podrían seguir un sistema de expresión de su RdRp basado en un mecanismo de terminación y reiniciación acoplados (Ghabrial y Nibert 2009). No obstante, TcV1 difiere del resto de miembros del género en que los dos ORFs (fases de lectura abierta) presentes en su genoma no solapan como ocurre en la mayoría de los victorivirus. Así, en TcV1 ambos ORFs están en la misma fase de lectura, y el codón de parada de ORF1 está directamente seguido por el codón de inicio de ORF2. Ahora bien, este tipo de organización es perfectamente compatible con el mecanismo de terminación y reiniciación acoplados que los victorivirus siguen para la expresión de su RdRp (Guo *et al.*, 2009).

En lo referente al ciclo de vida de estos dos virus, es muy probable que éste sea similar al desarrollado por la especie tipo del género, *Helminthosporium victoriae* virus 190S (HvV190S) (Ghabrial y Nibert 2009). No obstante, aunque se tienen ciertos datos moleculares sobre este proceso en HvV190S, en general, el estudio del ciclo de vida de los victorivirus continúa siendo un terreno bastante inexplorado. De este modo, teniendo en cuenta el ciclo descrito para HvV190S, los viriones maduros de TcV1 y BbRV1 podrían contener en el interior de su cápsida proteica una molécula de dsRNA correspondiente al genoma completo del virus y varias RdRp (Fig. 1). Dentro del virión tendría lugar la replicación del material genético del virus por medio de la RdRp, y a continuación esta proteína cambiaría su función replicativa a transcripcional. La transcripción en HvV190S parece ser asimétrica (sólo se generan cadenas de sentido positivo de RNA) y conservativa (la cadena de sentido positivo es retenida como parte del dúplex molde, mientras que las cadenas de sentido positivo de RNA de nueva formación son liberadas fuera del virión),

luego TcV1 y BbRV1 por semejanza, podrían llevar a cabo una transcripción de estas características. Una vez que las cadenas de RNA positivas son liberadas fuera del virión, tendría lugar la traducción proteica a cargo de la maquinaria del hongo hospedador. De modo que, sintetizadas las nuevas CP y RdRp, se produciría de nuevo el proceso de ensamblaje de los viriones, que empaquetarían una cadena de RNA de sentido positivo, varias RdRp e incluso alguna cadena defectiva de RNA o RNAs satélites, como ocurre en el victorivirus *Helicobasidium mompa* totivirus 1–17 (Nomura *et al.*, 2003). Ciertas modificaciones post-traduccionales en las CP podrían ocurrir tras el ensamblaje de los viriones como ocurre en HvV190S, donde las CP sufren procesos de fosforilación (Ghabrial, 2010). A continuación, se producirían los viriones maduros tras la síntesis por parte de la RdRp de las cadenas de sentido negativo de RNA para la formación del dúplex de dsRNA que constituye el genoma completo del virus. Por último, para cerrar el ciclo en HvV190S ocurren ciertos procesos de proteólisis en las CP que parecen estar relacionados con la liberación de las moléculas de RNA positivo fuera del virión (Ghabrial, 2010). Ahora bien, la localización subcelular donde este ciclo de vida tiene lugar aún sigue siendo un terreno bastante inexplorado.

Fig. 1. Hipotético ciclo de vida de TcV1 y BbRV1 basado en el ciclo de vida descrito para HvV190S. Esquema adaptado de Ghabrial (2010).



En cuanto a la biología del género *Victorivirus* al que pertenecen TcV1 y BbRV1, es importante resaltar que al igual que para el resto de micovirus descritos no se conocen en vectores para su transmisión (Ghabrial y Nibert, 2009). Así, los victorivirus se transmiten de forma intracelular: verticalmente durante la división del hospedador y en la esporogénesis; y horizontalmente mediante fenómenos de anastomosis hifal entre cepas compatibles del hongo hospedador (Ghabrial y Nibert, 2009). Ahora bien, aunque algunos de los victorivirus conocidos son excluidos en la formación de ascosporas, éstos son transmitidos a las esporas asexuales o conidios, siendo este medio su principal vía de diseminación. De acuerdo con este tipo de transmisión, cuando existen infecciones mixtas, todos los virus que componen la infección se transmiten a los conidios con la misma frecuencia (Romo *et al*, 2007; Tuomivirta y Hantula, 2005). Sin embargo, en esta tesis se describieron por primera vez frecuencias de transmisión diferentes para cada uno de los tres micovirus, TcV1, TcV2, y TcV3, que infectaban al hongo endofítico y entomopatógeno *T. cylindrosporum.*

TcV3 no fue identificado en esta memoria, pues no se consiguió ni un solo clon perteneciente a su genoma. Sin embargo, TcV2 fue parcialmente secuenciado, así, se consiguió la secuencia completa de uno de los dsRNAs que compondrían el genoma cuatripartito de TcV2. El dsRNA secuenciado codificaba para una RdRp similar a la de micovirus miembros de la familia *Chrysoviridae.* Por otra parte, los resultados del experimento de transmisión a las esporas asexuales de cada uno de los seis elementos de dsRNA que albergaba *T. cylindrosporum*, revelaron que cuatro de los elementos con tamaños similares a los de la familia *Chrysoviridae* (2.4-3.6 kbp), entre ellos el que codifica para la RdRp, siempre se transmitían juntos, o no se transmitían a las esporas asexuales. Lo cual reafirmó la idea de que TcV2 podría ser un nuevo miembro de la familia *Chrysoviridae*. Además, una purificación parcial de los micovirus que infectan a *T. cylindrosporum*, reveló que los 4 dsRNAs que supuestamente componen el genoma de TcV2 están encapsidados, lo cual también concuerda con el hecho de que TcV2 sea un posible miembro de la familia *Chrysoviridae.*

3.3. Naturaleza dual de *T. cylindrosporum*: acaricida y endofito.

T. cylindrosporum ha sido una de las especies de hongos en que se ha centrado esta tesis doctoral. Este hongo entraña un doble interés, ya que además de ser un hongo entomopatógeno, las cepas objeto de estudio tienen naturaleza endofítica. De este modo, también quisieron estudiarse estos dos aspectos inherentes a la biología de *T. cylindrosporum*, patogenicidad y endofitismo.

T. cylindrosporum es un conocido patógeno de insectos, como por ejemplo mosquitos de los géneros *Aedes*, *Culex* y *Anopheles*, los cuales se caracterizan por ser vectores de parásitos que producen malaria, dengue o fiebre amarilla (Scholte *et al.*, 2004). En este trabajo se probó su capacidad como acaricida contra dos especies de garrapatas *Ornithodoros erraticus* y *Ornithodoros moubata*, las cuales son vectores de patógenos porcinos y humanos.

Cinco cepas de *T. cylindrosporum* aisladas de los distintos hábitats que el hongo es capaz de colonizar, suelo, insecto y el interior de hojas de gramínea, fueron usadas en los ensayos de patogenicidad. Además, dos de las cepas estaban infectadas por virus y las otras tres estaban libres de infección. Ambas especies de garrapatas resultaron ser susceptibles a todas las cepas de *T. cylindrosporum* incluidas en el ensayo. También se observaron diferentes porcentajes de mortalidad entre los distintos estadíos de desarrollo de las garrapatas. Los porcentajes de mortalidad alcanzados después de un mes de la inoculación con cada una de las cepas de *T. cylindroporum* en estudio, fueron próximos al 60% para *O. erraticus.* Estos porcentajes de mortalidad son similares a los obtenidos con dos hongos que se están comercializando como acaricidas, *B. bassiana* y *M. anisopliae* (Fernandes *et al.*, 2003; Ostfeld *et al.*, 2006; Samish *et al.*, 2004). Los resultados obtenidos en este estudio indican que *T. cylindrosporum* podría usarse también con este fin, y además al ser un hongo con amplio espectro de hospedadores, podría aplicarse en establos para controlar al mismo tiempo garrapatas y otros insectos patógenos del ganado (Polar *et al.*, 2008). Adicionalmente, algunas de las cepas de *T. cylindrosporum* del estudio mostraron resistencia a las altas temperaturas, así, estos aislados podrían tener una mejor actuación en ambientes cálidos. De hecho, esta sería una cualidad a tener en cuenta en la elección de la cepa a aplicar según las características del medio (Quesada-Moraga *et al.*, 2006b).

En lo referido al estudio de la capacidad endofítica de *T. cylindrosporum*, dos de las cepas de *T. cylindrosporum* utilizadas en esta tesis fueron aisladas como endofíos de dos especies de gramíneas, *Festuca rubra* y *Holcus lanatus*. Otros hongos entomopatógenos

como *B. bassiana*, *M. anisopliae*, *L. lecanii*, *Paecilomyces* spp. o *Cladosporium* spp. han sido aislados como endofitos de distintas especies de plantas, monocotiledóneas y dicotiledóneas (Vega *et al.,* 2008). Así, la capacidad endofítica de algunos hongos entomopatógenos está despertando un gran interés por su potencial uso en agricultura como biocontroladores. Además, estos hongos no solo podrían aplicarse contra plagas de insectos fitófagos, sino también contra hongos y bacterias fitopatógenas, ya que se ha descubierto que algunos de los metabolitos que producen tienen actividad antifúngica, y algunos de estos hongos tienen la capacidad de inducir respuestas sistémicas en plantas contra infecciones bacterianas (Ownley *et al.,* 2010; Vega *et al*., 2008). Por todo ello, en esta memoria se quiso comprobar la capacidad endofítica de *T. cylindrosporum* inoculándolo artificialmente en plantas de tomate y judía.

Las dos cepas empleadas en el ensayo fueron capaces de penetrar en ambas especies vegetales y establecerse como endofitos, puesto que ambas cepas se reaislaron 35 días después de su inoculación de plantas de tomate y de judía. De este modo, los resultados obtenidos para la inoculación artificial de *T. cylindrosporum* son comparables a los obtenidos con otras especies entomopatógenas como *B. bassiana*. De hecho esta especie ha sido inoculada artificialmente con éxito en especies como amapolas del opio, bananos, palmera datilera, café, maíz, patata o cacao (Ownley y Griffin, 2008). De la misma forma, *T. cylindrosporum* podría tener también un amplio espectro de hospedadores, ya que fue originalmente aislado de gramíneas pero ha sido exitosamente inoculado en plantas dicotiledóneas como son el tomate y la judía. De modo que *T. cylindrosporum* podría ser un excelente candidato a tener en cuenta en sucesivos estudios sobre biocontroladores endofíticos y entomopatógenos.

3.4. Efecto de los micovirus en *T. cylindrosporum.*

En esta tesis también se estudió el efecto que algunos micovirus producían en su hospedador. Para ello se eligieron los virus asociados a *T. cylindrosporum*, abordándose el estudio de las relaciones virus-hongo desde dos perspectivas: *T. cylindrosporum* entomopatógeno y *T. cylindrosporum* endofito, puesto que las cepas de las que se disponía tienen esta doble naturaleza. Hasta la fecha sólo existen dos trabajos que atribuyan a un micovirus la producción de un efecto en su hospedador entomopatógeno, hipervirulencia, en *M. anisopliae* (Melzer y Bidochka, 1998), e hipovirulencia en *B. bassiana* (Dalzoto *et*

al., 2006). No obstante, los miembros del género *Victorivirus*, entre los que se encuentra TcV1, aunque están asociados con infecciones persistentes, éstas son asintomáticas. Así, a pesar de que varios de los hospedadores de este grupo de virus son hongos fitopatógenos, no existen casos documentados en los que la patogenicidad del hongo hospedador sea positiva o negativamente modulada por la infección de un victorivirus (Ghabrial y Nibert, 2009).

En el estudio sobre la capacidad como acaricida de *T. cylindrosporum* se utilizaron como ya comentamos dos cepas infectadas por micovirus y tres libres de infección. No se detectaron efectos significativos claros atribuibles a la carga vírica de las distintas cepas de *T. cylindrosporum* en estudio, ni en su patogenicidad hacia las dos especies de garrapatas, ni en su respuesta a altas temperaturas. No obstante, cuando se realizó este bioensayo no se disponía de cepas isogénicas de *T. cylindrosporum*, con y sin infecciones víricas, para poder atribuir más claramente a un virus determinado un efecto en la patogenicidad o en la resistencia a altas temperaturas de *T. cylindrosporum*.

Posteriormente, en el estudio de caracterización molecular de los micovirus asociados a cepas endofíticas de *T. cylindrosporum* se obtuvieron cepas isogénicas del hongo que albergaban diversas combinaciones de los virus TcV1, TcV2 y TcV3, o cepas libres de infección. Partiendo de este material sería posible probar si alguno de estos virus tiene alguna implicación en la patogenicidad de *T. cylindrosporum* contra *O. erraticus* u *O. moubata.*

En un estudio sobre el efecto que TcV1, TcV2 y TcV3 tenían en la respuesta a altas temperaturas de las dos cepas endofíticas de *T. cylindrosporum* (11 y 3398M), no se observó un efecto claro de los virus en la resistencia a las altas temperaturas. No obstante, si sólo se hubiera incluido una de las cepas en el estudio si podrían haberse sacado conclusiones sobre el efecto virus. Por ejemplo, para el aislado 3398M, parece que existe un efecto significativo producido por la combinación de los virus TcV2 y TcV3. Así las cepas infectadas con esta mezcla de virus crecen mejor a 22 ºC y en condiciones de estrés térmico (29 ºC) que las cepas libres de infección. No obstante, los efectos producidos en una u otra cepa por un mismo virus en ocasiones son contrarios y por ello es difícil atribuir un efecto claro a un virus determinado. En vista de estos resultados, habría que tener cierto escepticismo respecto a afirmaciones sobre los efectos de micovirus basados en aislados isogénicos de una única cepa. Por ejemplo, dos trabajos concluyeron que la patogenicidad de *B. bassiana* y *M. anisopliae* contra distintos insectos es afectada por la presencia de un

micovirus, produciendo hipovirulencia en *B. bassiana*, e hipervirulencia en *M. anisopliae* (Dalzoto *et al.*, 2006; Melzer y Bidochka, 1998). En ambos casos solo se utilizaron aislados isogénicos infectados y libres de infección provenientes de una única cepa del hongo.

Por otro lado, también se quiso ver que relación guardaba la infección por TcV1 con la capacidad endofítica de su hospedador, *T. cylindrosporum*. Así, se observó una interacción significativa entre el tipo de infección por micovirus y la especie de planta hospedadora del hongo. De este modo, en judía las cepas de *T. cylindrosporum* infectadas por TcV1 eran capaces de penetrar mejor en las plantas que las cepas sin virus. En cambio, en tomate se observó exactamente lo contrario, es decir, que las cepas libres de virus penetraban mejor en las plantas.

Los resultados obtenidos en esta tesis indican la existencia de una gran diversidad de especies de micovirus en distintas especies de hongos endofíticos y entomopatógenos. De este modo, debido al potencial que estos dos grupos de hongos entrañan por su uso como biocontroladores, como armas de mejora de las características agronómicas de plantas de interés para el hombre, o como productores de metabolitos de importancia en medicina, el estudio de las relaciones micovirus-hongo, podrían ayudar a entender mucho mejor las relaciones hongo-planta u hongo-insecto. Así, el estudio de estos triángulos simbióticos puede llevar en el futuro a una mejora de estos recursos naturales tan importantes en la lucha contra plagas y patógenos vegetales, en la mejora de las cosechas e incluso en la producción de metabolitos de interés médico.

4.CONCLUSIONES

1. Los micovirus son relativamente comunes en especies de hongos endofíticos de gramíneas. La incidencia observada entre especies (22.6%) podría ser una infraestimación, puesto que de muchas especies solo se analizó un aislado.

2. La incidencia de micovirus entre las especies entomopatógenas *Tolypocladium cylindrosporum* y *Beauveria bassiana* es elevada obteniéndose valores de incidencia de micovirus entre aislados cercanos al 50% o superiores.

3. La diversidad de virus observada para los aislados analizados de *B. bassiana* procedentes de diversos hábitats fue muy alta, llegándose a obtener hasta 27 perfiles diferentes de dsRNA entre los 50 aislados infectados. No obstante, esta diversidad de micovirus en *B. bassiana* podría ser mayor, ya que se comprobó que un mismo perfil de dsRNA no siempre se corresponde con una infección por la misma especie de virus.

4. El virus más común encontrado entre los aislados de *B. bassiana* analizados fue completamente secuenciado e identificado. *Beauveria bassiana* RNA virus 1 (BbRV1) constituye un nuevo miembro del género *Victorivirus* (Familia: *Totiviridae*).

5. En *T. cylindrosporum* se detectaron tres micovirus diferentes: *Tolypocladium cylindrosporum* virus 1 (TcV1), *Tolypocladium cylindrosporum* virus 2 (TcV2) y *Tolypocladium cylindrosporum* virus 3 (TcV3). TcV1 fue totalmente secuenciado e identificado y constituye un nuevo miembro del género *Victorivirus* (Familia: *Totiviridae*); TcV2 fue parcialmente secuenciado y es muy probable que constituya un nuevo miembro de la familia *Chrysoviridae*. La eficiencia de transmisión a las esporas asexuales de TcV1, TcV2 y TcV3 es variable dependiendo del virus. Así, las frecuencias de transmisión a las esporas asexuales de estos tres virus varían entre 0% y 90%.

6. *T. cylindrosporum* es un patógeno de *O. erraticus*, cuatro de las cinco cepas analizadas de dicho hongo producen mortalidades entre los distintos estadíos de desarrollo de esta especie de garrapata cercanas al 70%. De este modo, *T. cylindrosporum* podría ser un buen acaricida.

7. *T. cylindrosporum* es capaz de penetrar en plantas de tomate y judía y establecerse con éxito como endofito, reaislándose hasta cinco semanas después de su inoculación. Además, *T. cylindrosporum* es capaz de comportarse como generalista en cuanto a la especie hospedadora ya que fue aislado originalmente como endofito de la gramínea *Festuca rubra.* Así, *T. cylindrosporum* podría usarse como agente de control biológico de distintas plagas de insectos fitófagos o de insectos vectores de patógenos vegetales.

8. La presencia de micovirus no produce un efecto claro en la capacidad endofítica de *T. cylindrosporum*, en su patogenicidad contra garrapatas o en la resistencia a las altas temperaturas de dicho hongo.

5. APÉNDICE

Apéndice 1. Secuencia de nucleótidos del genoma completo de *Tolypocladium cylindrosporum* virus 1 (TcV1)

> TcV1

tgaaattcaccgcccgcgaatcaccgattcccgagcgtaccacgggccccccgcactgcc
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```

--- Codón de inicio
--- Codón de parada

ORF 1 – Proteína de cápsida (CP)

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372 tctcctcgcggtgggagcctcgataacgacaatcgttttcgtcgt
    S  P  R  G  G  S  L  D  N  D  N  R  F  R  R
417 tatcgtacttccgttcgcacgaccgccaccattggcggtaacgat
    Y  R  T  S  V  R  T  T  A  T  I  G  G  N  D
462 gactcgcgcaccacctctatcttctacgaagtggggcgtgcggtc
    D  S  R  T  T  S  I  F  Y  E  V  G  R  A  V
507 aacacgaagggccgcgcccttaaccgcccctccgatggcgccctg
    N  T  K  G  R  A  L  N  R  P  S  D  G  A  L
552 tgcatcgaagcggcttatcccacgaatactgtcctagctgaggat
    C  I  E  A  A  Y  P  T  N  T  V  L  A  E  D
597 ttcattggtctggccaagaaatacaccaacttctctgcctcgttc
    F  I  G  L  A  K  K  Y  T  N  F  S  A  S  F
642 gagtattcatcgctcgctggtgtcgccgagcgtctcgcccgcgcg
    E  Y  S  S  L  A  G  V  A  E  R  L  A  R  A
687 cttgccgcgtccagcgtcttcacggacgtcgactcgaacgacatc
    L  A  A  S  S  V  F  T  D  V  D  S  N  D  I
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 732 cgcggcggcgccgggctggtcgtcaacgctgttggtacttacgac
     R  G  G  A  G  L  V  V  N  A  V  G  T  Y  D
 777 ggccccatctcctcgctcaccaacacggtctacatacctcgcctc
     G  P  I  S  S  L  T  N  T  V  Y  I  P  R  L
 822 gtcaactcctctatcaccggtgaagtcttctcggtcctggcgaac
     V  N  S  S  I  T  G  E  V  F  S  V  L  A  N
 867 gctgtatctggtgaaggctcctccgttgcgaccgacataatcgaa
     A  V  S  G  E  G  S  S  V  A  T  D  I  I  E
 912 ctcgacgcaggtacccgccaacccctcatacctgaggttgacccc
     L  D  A  G  T  R  Q  P  L  I  P  E  V  D  P
 957 gctggactcgctgccgcctgtgttgacgcccttcgcatcgtcggt
     A  G  L  A  A  A  C  V  D  A  L  R  I  V  G
1002 tctaatatgattgcaagcgaccagggcccgctattctccctcgcc
     S  N  M  I  A  S  D  Q  G  P  L  F  S  L  A
1047 gttacccgtggtatccaccgagtcctttcagtcgttggccacacc
     V  T  R  G  I  H  R  V  L  S  V  V  G  H  T
1092 gacgagggtggcatcacccgcgacttgttacggtgctcgtctttc
     D  E  G  G  I  T  R  D  L  L  R  C  S  S  F
1137 gccccgcctttcggcggcatccactatgctctcgagccttatgca
     A  P  P  F  G  G  I  H  Y  A  L  E  P  Y  A
1182 gggttgcccgccctccaatttaccgcgttccccgcctacgccgcc
     G  L  P  A  L  Q  F  T  A  F  P  A  Y  A  A
1227 tacgtggattccatcgccctaactaccgccgctgtcgtcgcacac
     Y  V  D  S  I  A  L  T  T  A  A  V  V  A  H
1272 gctgaccccggtgtcatgtacggcggctcttggttcccaaccttc
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1317 tacaatggcactgccgacagcgatggcaccgttcgccctggccag
     Y  N  G  T  A  D  S  D  G  T  V  R  P  G  Q
1362 aatcaggccgggacggcagccatggccaatcgtaaccgtgcccag
     N  Q  A  G  T  A  A  M  A  N  R  N  R  A  Q
1407 ctcttgggctccgcgccggccttttttcagtgaatacatccgcggc
     L  L  G  S  A  P  A  F  F  S  E  Y  I  R  G
1452 ctcgcaactatctttggtgcgtcgggtgactcctccctcgcctgc
     L  A  T  I  F  G  A  S  G  D  S  S  L  A  C
1497 cgctttatgggcgcggcctcttatgctctcccgcaggatccgcgc
     R  F  M  G  A  A  S  Y  A  L  P  Q  D  P  R
1542 catctccgatacgctaccgtgtccccctggttctggatagagccg
     H  L  R  Y  A  T  V  S  P  W  F  W  I  E  P
1587 accggtctgctgccgcacgacttccttggctctaaggccgaggcg
     T  G  L  L  P  H  D  F  L  G  S  K  A  E  A
1632 aacggcgcggggtcattcgcttggaaggacaccacccgtactagg
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1677 gtcgcttgggacgaactaacccagcacggtgaggccgacaccacc
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1722 ttcagtgcatacgtcgccaggttcaggtctcctcgtcagcagtgg
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1767 ttcttcgcgcactggatgaaccatcccctcaacggcctcggcgct
     F  F  A  H  W  M  N  H  P  L  N  G  L  G  A
1812 atccgagtccggcagctcgatcctaacgggatcgtgcagccagga
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1857 cagtgcgtcgcgcaccccgacgtccgtgaccgtgttgaagccgac
     Q  C  V  A  H  P  D  V  R  D  R  V  E  A  D
1902 ctacctttcacagactacctatggacacgcggccaatcgcccccc
     L  P  F  T  D  Y  L  W  T  R  G  Q  S  P  P
1947 cccgcccccggcgagcttctcaacttgagcgggtctgttggcttc
     P  A  P  G  E  L  L  N  L  S  G  S  V  G  F
1992 ctcgtccgccatctaacttatgatgacgacggcgtcccgcatgaa
     L  V  R  H  L  T  Y  D  D  D  G  V  P  H  E
2037 gaacatgtcccaacgcgccgcgaatttctcgacaccacagtgacg
     E  H  V  P  T  R  R  E  F  L  D  T  T  V  T
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2127 gcgggcgacaaccacgtccggcgtgccaggaccaaggctgctaac
     A  G  D  N  H  V  R  R  A  R  T  K  A  A  N
2172 gaactagctgccgctaggcgtcgtgccgctgtctttggtcgtgcc
     E  L  A  A  A  R  R  R  A  A  V  F  G  R  A
2217 gacgtggctgagatgcccatactgactagcgcgcccgctctagcc
     D  V  A  E  M  P  I  L  T  S  A  P  A  L  A
2262 ccgccccccagcgccggtcttgagcgcggtagtgatcctggcggt
     P  P  P  S  A  G  L  E  R  G  S  D  P  G  G
2307 agcggctcccagcgtcgtgccgtcccggccggtttgcccggaaac
     S  G  S  Q  R  R  A  V  P  A  G  L  P  G  N
2352 gacacttgggcgcgggagcctaccggcgctcctcgcaatcccgtc
     D  T  W  A  R  E  P  T  G  A  P  R  N  P  V
2397 ccccaccaccagcctctccgggctcctcagctcgcgcgtcaagcc
     P  H  H  Q  P  L  R  A  P  Q  L  A  R  Q  A
2442 ggtggtctaggtggcggcgccgcccctatccccccccctccacct
     G  G  L  G  G  G  A  A  P  I  P  P  P  P  P
2487 ggtgtccccgccccgcctgcattgccccccgctgatgacgataac
     G  V  P  A  P  P  A  L  P  P  A  D  D  D  N
2532 gctcctccagctcctgtggccactgctccccctgttgccgacccc
     A  P  P  A  P  V  A  T  A  P  P  V  A  D  P
2577 caggctatggtgggcgaccaggtctaa 2603
     Q  A  M  V  G  D  Q  V  *
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ORF 2 – RNA polimerasa dependiente de RNA (RdRp)

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2649 tcctacctacacgacttactccccgagttgggtgacgtgactgaa
     S  Y  L  H  D  L  L  P  E  L  G  D  V  T  E
2694 tcttatgctgatctcgagttgaccgagcagctgattaagtttgca
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2739 acccactcagtgtctctccgcggtgtacatcctctcggtcctgtt
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2784 gcggtctcactactatgtgtggcctaccccgtccagtgtgatttc
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     R  L  S  D  L  T  A  V  L  R  H  S  F  S  F
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     R  R  V  T  L  R  K  A  P  A  T  I  G  A  L
2964 cacactaaagtcgcgcgtctttgtaactccgatagcgcggcgcgt
     H  T  K  V  A  R  L  C  N  S  D  S  A  A  R
3009 tcccagttgtggccacccaaacgccacgccgcagctggtacgaag
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3054 gttaatatccgcttagcacccctgccgtcgccttgtcacgcagc
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     Y  G  P  A  W  L  G  R  A  L  T  P  L  V  G
3144 tgcgcggaagacgcagtttgctgtgcacttctcctcgcgactgcg
     C  A  E  D  A  V  C  C  A  L  L  L  A  T  A
3189 ctcgagccgcacttcgggcacctgggactcgaggtggcccagtct
     L  E  P  H  F  G  H  L  G  L  E  V  A  Q  S
3234 atgatcctcgagccgaacaacgctaagggcctgtctaacgctctc
     M  I  L  E  P  N  N  A  K  G  L  S  N  A  L
```

```
3279 aaggcccttggctggaactccactctccccggctcgatgctggtt
     K  A  L  G  W  N  S  T  L  P  G  S  M  L  V
3324 gagggcggggcactccaaggccgcggtgtatcgcccgttgacatg
     E  G  G  A  L  Q  G  R  G  V  S  P  V  D  M
3369 gacgccgagatcgcctcgcgtactacgcctgacctcgtcactgac
     D  A  E  I  A  S  R  T  T  P  D  L  V  T  D
3414 atggtcatatcctcggctgctgagatggctcctcacatccgcgct
     M  V  I  S  S  A  A  E  M  A  P  H  I  R  A
3459 atactggctctcgagcttcccagcaactccggtttgggagacctc
     I  L  A  L  E  L  P  S  N  S  G  L  G  D  L
3504 gacgaattctggtccgcacggtggttatggtgtgttaacggtgcg
     D  E  F  W  S  A  R  W  L  W  C  V  N  G  A
3549 caaaatcgtgcatccgaccgctctctcaacttgccctcccccgtg
     Q  N  R  A  S  D  R  S  L  N  L  P  S  P  V
3594 cgcggcgcgaaacgttatcggcgcatggctgctgaggaggtcagt
     R  G  A  K  R  Y  R  R  M  A  A  E  E  V  S
3639 tcgaaccccatatacgactgggacggcacgacgaacgtgtctgcc
     S  N  P  I  Y  D  W  D  G  T  T  N  V  S  A
3684 tcaaccaagttggagccggggaagtcgcgcgccatcttcgcgtgc
     S  T  K  L  E  P  G  K  S  R  A  I  F  A  C
3729 gacacctcgtcgtacttcgccttctcgtggatactcgatcgcgcc
     D  T  S  S  Y  F  A  F  S  W  I  L  D  R  A
3774 cagcgtgactggcgcggacaccgtgtcctccttaacccgggagag
     Q  R  D  W  R  G  H  R  V  L  L  N  P  G  E
3819 gggggcctctacggcgtagcacggcgcgtcaaaggtgcccagggc
     G  G  L  Y  G  V  A  R  R  V  K  G  A  Q  G
3864 cgtggcggcgtcaacgtcatgctcgactacgacgacttcaattcg
     R  G  G  V  N  V  M  L  D  Y  D  D  F  N  S
3909 caccactccctcggcgtccagcaggaactcactcgccagctctgc
     H  H  S  L  G  V  Q  Q  E  L  T  R  Q  L  C
3954 caactctacaacgctccctcctggtataccagcgtcctcgtggac
     Q  L  Y  N  A  P  S  W  Y  T  S  V  L  V  D
3999 tccttcgaccgcatgtacatcacccaccgcgggtctcgtaaacgc
     S  F  D  R  M  Y  I  T  H  R  G  S  R  K  R
4044 attctcggcacactgatgtccgggcaccgcggcacatcattcatc
     I  L  G  T  L  M  S  G  H  R  G  T  S  F  I
4089 aactccgtgctgaacgccgcgtacatacgtgccgccgtgggtggt
     N  S  V  L  N  A  A  Y  I  R  A  A  V  G  G
4134 gctttctttgatcgcctcgtctcgctgcacgcgggcgatgacgct
     A  F  F  D  R  L  V  S  L  H  A  G  D  D  A
4179 tacatgcgttgcagcacgcttgccgaggcagcccacgtcctaact
     Y  M  R  C  S  T  L  A  E  A  A  H  V  L  T
4224 cgctgtgcccgtttcgggtgccgtatgaatccgacaaaacagagt
     R  C  A  R  F  G  C  R  M  N  P  T  K  Q  S
4269 attggtttccggcatgccgagttcctcaggctcggaattggtgac
     I  G  F  R  H  A  E  F  L  R  L  G  I  G  D
4314 aaatatgccgtcggttacctttgccgctcgatatctactctggtt
     K  Y  A  V  G  Y  L  C  R  S  I  S  T  L  V
4359 gcgggatcgtgggttgccccggacccctgagccccgaggacggc
     A  G  S  W  V  A  P  D  P  L  S  P  E  D  G
4404 cttacttccgcgatcacgacagtccgcagttgtattaaccgcggg
     L  T  S  A  I  T  T  V  R  S  C  I  N  R  G
4449 tgtccctcttctctctcccgtatcatcgcgaggacgtactcgtcc
     C  P  S  S  L  S  R  I  I  A  R  T  Y  S  S
4494 atacatggctacccctccgtgttttggatgcattacttagcggt
     I  H  G  Y  P  L  R  V  L  D  A  L  L  S  G
4539 gccgcctgccttgaatcaggcgcggtctacaacacggactatacc
     A  A  C  L  E  S  G  A  V  Y  N  T  D  Y  T
4584 ttacgccagtatcgtgttgtccggcccctccccgatgacctcccg
     L  R  Q  Y  R  V  V  R  P  L  P  D  D  L  P
```

```
4629 ctccctccgcaacacagggagtacgccacccgcgagtaccttgcc
     L  P  P  Q  H  R  E  Y  A  T  R  E  Y  L  A
4674 aatcacgtctcacccatagaggcgcgggcagcacaacttgcgtcg
     N  H  V  S  P  I  E  A  R  A  A  Q  L  A  S
4719 gcagatttggtgcggatcatggtgcggagcagctactccaaggga
     A  D  L  V  R  I  M  V  R  S  S  Y  S  K  G
4764 gtaacacgtcatccatcatcccccacgtcgcgccgtcgtcctcaa
     V  T  R  H  P  S  S  P  T  S  R  R  R  P  Q
4809 cttgtgtctttacccactgtcaaggcaactggtttcaccacagct
     L  V  S  L  P  T  V  K  A  T  G  F  T  T  A
4854 gctgaactcgcaaaacgtcaacctccggtcgggaagcttgctaac
     A  E  L  A  K  R  Q  P  P  V  G  K  L  A  N
4899 ttcccgttgctccggttaatcgaggcccggctcactgacgaccag
     F  P  L  L  R  L  I  E  A  R  L  T  D  D  Q
4944 atcagtgagttactatcattcaatgggacccctcctggtggtctg
     I  S  E  L  L  S  F  N  G  T  P  P  G  G  L
4989 cctccccgcatagcagccttcggtgttgaaggccattgctgtaac
     P  P  R  I  A  A  F  G  V  E  G  H  C  C  N
5034 gtcatagggtacctaccctactctgacgcatgctcatacagcaag
     V  I  G  Y  L  P  Y  S  D  A  C  S  Y  S  K
5079 cgtacgacctgcgataacattatcgccggttactcagtctattcc
     R  T  T  C  D  N  I  I  A  G  Y  S  V  Y  S
5124 tag 5126
     *
```

Apéndice 2. Secuencia completa de nucleótidos de dsRNA3. RNA polimerasa dependiente de RNA (RdRp) de *Tolypocladium cylindrosporum* virus 2

>TcV2- RdRp

cgcaaaaaagagataaaggtatcaatttttgcacactaccagaaatggctattaaattt
tctacggctagcgcgttctcagaacagaaagcaacaatgggtgcatcgtacattggtgag
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cttcacatgcatgcagttgtgctgccagtgttatgcggaaagagtctgatgtgccacaag
ttcagtggatatgacattgacgacatcgtggtgaatgaggaagcattcaagtgcgatcaa
gagtgggaagagatgattgacgcacgggaacggggctatttcggtggcgataactccggc
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gggattcacacattggtccgcgacactaagtcgatgctcggtgcggctctaaacactcat
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caaatattaagcgatgcgcgagtgccgcctactgtagtgaacatcacgggtgtaaaatca
ataagacgcgagcattacgataggcatgggcatgatgtgcacgtgttcaggcgagattac
gctcggttaaagcacaggaccgacgcgcttaaaaacgctggcgcacgatacgacgcattg
gcagcagcggtgaagcctggttacagaagacgattggcacatgttgtaggcctcacatat

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gcggttgatggtgatctactatactactggaaggagaatttaactcttttcggctgcggc
acatacttactaacggaagattactacaacgctgtgcagttgatggctttagtgacggca
ccaggctacagtgacaatgagataagcgagcgattagcatactacgcgactgcgcttgct
aacagcaatatgatgaattactagagcgtcatagtgcgaagacaggaattcaaaagaatt
tctacc
```

--- Codón de inicio
--- Codón de parada

ORF 1 – RNA polimerasa dependiente de RNA (RdRp)

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    46 atggctattaaattttctacggctagcgcgttctcagaacagaaa
       M  A  I  K  F  S  T  A  S  A  F  S  E  Q  K
    91 gcaacaatgggtgcatcgtacattggtgagccgtcaggcgcaggc
       A  T  M  G  A  S  Y  I  G  E  P  S  G  A  G
   136 acactattgatgccaaatgagcgagaaaggatcggggtcggcaaa
       T  L  L  M  P  N  E  R  E  R  I  G  V  G  K
   181 cttcacatgcatgcagttgtgctgccagtgttatgcggaaagagt
       L  H  M  H  A  V  V  L  P  V  L  C  G  K  S
   226 ctgatgtgccacaagttcagtggatatgacattgacgacatcgtg
       L  M  C  H  K  F  S  G  Y  D  I  D  D  I  V
   271 gtgaatgaggaagcattcaagtgcgatcaagagtgggaagagatg
       V  N  E  E  A  F  K  C  D  Q  E  W  E  E  M
   316 attgacgcacgggaacggggctatttcggtggcgataactccggc
       I  D  A  R  E  R  G  Y  F  G  G  D  N  S  G
   361 tacctcacatcaaatcgcataatgctacggcgggcgcgtaggttt
       Y  L  T  S  N  R  I  M  L  R  R  A  R  R  F
   406 ttcgacttgtttgtgggggatggcaacgcgcctgtgttatacgtg
       F  D  L  F  V  G  D  G  N  A  P  V  L  Y  V
   451 caaacagccgagatggcagaggcacttggagcagagatcatctat
       Q  T  A  E  M  A  E  A  L  G  A  E  I  I  Y
   496 gtgggtagtgttgctcgtggagtggccgcggcgacgccacgttat
       V  G  S  V  A  R  G  V  A  A  A  T  P  R  Y
   541 caggcactgtctgacgaggagcaacgtttgatgctgaagcttctg
       Q  A  L  S  D  E  E  Q  R  L  M  L  K  L  L
   586 atagagcaggacaatgctaatgaggtgtattgcgcacgacacgga
       I  E  Q  D  N  A  N  E  V  Y  C  A  R  H  G
   631 ctacagtatgacggcatatctaacggttacgaggtacaggcacaa
       L  Q  Y  D  G  I  S  N  G  Y  E  V  Q  A  Q
   676 cgtgtatcggaacggttgcgtcgctgcggtttgaacgcattgacg
       R  V  S  E  R  L  R  R  C  G  L  N  A  L  T
   721 tcagcatcgcaagacgcgcttgacaggctataccttgagacttct
       S  A  S  Q  D  A  L  D  R  L  Y  L  E  T  S
   766 tttcgtgggcggctgaatcaatcatgtgcgattgcatgtaacgca
       F  R  G  R  L  N  Q  S  C  A  I  A  C  N  A
   811 gagttaactgggtgggtcagggctgtggccgcccgtgaggcgcaa
       E  L  T  G  W  V  R  A  V  A  A  R  E  A  Q
   856 gtgcttgtcggcgctgcgttaccacaaggtgcgggccaagtgcat
       V  L  V  G  A  A  L  P  Q  G  A  G  Q  V  H
   901 aatcatgtgcgatgggctcgagtgatacacgctctggaacagcat
       N  H  V  R  W  A  R  V  I  H  A  L  E  Q  H
   946 gcactagcagttgttccgggccctaagcctgtgcgcaccagatgg
       A  L  A  V  V  P  G  P  K  P  V  R  T  R  W
   991 agcgagaagtttccatacggaccagggaatgctaaattcgctctc
       S  E  K  F  P  Y  G  P  G  N  A  K  F  A  L
  1036 gccaagataggtgactttctcgaatccgtatcggagagggattat
       A  K  I  G  D  F  L  E  S  V  S  E  R  D  Y
  1081 caaaatgggtacgcgtggttccggcaactactgaaccgtgatgat
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1171 tggtgctatgtcgcccccgagttgaaaccgctcgtggaaagtctc
     W  C  Y  V  A  P  E  L  K  P  L  V  E  S  L
1216 agaataggcggcttagctccattggtgttcgctgaggtgtgtaaa
     R  I  G  G  L  A  P  L  V  F  A  E  V  C  K
1261 gggattcacacattggtccgcgacactaagtcgatgctcggtgcg
     G  I  H  T  L  V  R  D  T  K  S  M  L  G  A
1306 gctctaaacactcatggactagcgctgtgtacgtacttcgattgt
     A  L  N  T  H  G  L  A  L  C  T  Y  F  D  C
1351 ctcgctggccgctacttcggtgaaggcgatatagagaaggagata
     L  A  G  R  Y  F  G  E  G  D  I  E  K  E  I
1396 catgaccgtacggtgtcgcttgagcctcggcactttataatgcct
     H  D  R  T  V  S  L  E  P  R  H  F  I  M  P
1441 gatggcagcaaatccgaggctgagtttgacatgcggttcaagaat
     D  G  S  K  S  E  A  E  F  D  M  R  F  K  N
1486 gctgtagctgatgtgttgcactccacactagttgacggtggtgct
     A  V  A  D  V  L  H  S  T  L  V  D  G  G  A
1531 cgcatattacgagccagcaaaataacaaagagctttgacacgttc
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1576 ttggagcaccgtaaagcgtgggtgaggcctgggtctgtaactggc
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1621 agcccgaaaactgatgtgtatatcaaggtggtcggtgatagagag
     S  P  K  T  D  V  Y  I  K  V  V  G  D  R  E
1666 atggggatacgcgaggttgcggatgatctgcatatgatgggtacg
     M  G  I  R  E  V  A  D  D  L  H  M  M  G  T
1711 tatgtgctatctagagttagacttaacaaggctgctacctttgag
     Y  V  L  S  R  V  R  L  N  K  A  A  T  F  E
1756 tttgagaagtttccggaactggtgcgcgattgcataggtgattac
     F  E  K  F  P  E  L  V  R  D  C  I  G  D  Y
1801 gtgcccaatagcttcacacgacacttcataaagaacgagattgcg
     V  P  N  S  F  T  R  H  F  I  K  N  E  I  A
1846 aaagtgaagggtcgagcattgttcccgtctcacgttgtgcactac
     K  V  K  G  R  A  L  F  P  S  H  V  V  H  Y
1891 atcgttgggacatatgtgctccaactactcatgaaggcagcgcct
     I  V  G  T  Y  V  L  Q  L  L  M  K  A  A  P
1936 attgaacacgcacgactgataccggatgaggcaacaccgagagac
     I  E  H  A  R  L  I  P  D  E  A  T  P  R  D
1981 gagcactggatgtggatggaagcgcgtgacttcaccgtagggctg
     E  H  W  M  W  M  E  A  R  D  F  T  V  G  L
2026 atgctcgattacgacgacttcaacgaaagccacgaaattcgcgac
     M  L  D  Y  D  D  F  N  E  S  H  E  I  R  D
2071 atgcagatgattattaattcgcttaagggcgtttaccgcagggcg
     M  Q  M  I  I  N  S  L  K  G  V  Y  R  R  A
2116 ggtgctttaagccccgatctgtcggctatgatagattgggtggtg
     G  A  L  S  P  D  L  S  A  M  I  D  W  V  V
2161 gaagcgtacgaaaagatggtgtttgaattcgacgggaagcagtac
     E  A  Y  E  K  M  V  F  E  F  D  G  K  Q  Y
2206 cacttcctgcatggcatgctgtcagggcaagcacctacgtcaatg
     H  F  L  H  G  M  L  S  G  Q  A  P  T  S  M
2251 ataaacacagtcatcaacaccgcaaacaaaagggttatccgcgag
     I  N  T  V  I  N  T  A  N  K  R  V  I  R  E
2296 caaatatttgcgctatttggtgagagcgtgatgacaaaaaggact
     Q  I  F  A  L  F  G  E  S  V  M  T  K  R  T
2341 tcaggggcgacgatgttgctgcggagacatatgatgttttccag
     S  G  G  D  D  V  A  A  E  T  Y  D  V  F  Q
2386 gcagcgatgatagtgaaagtcggggagatgatgggcttcgcgttc
     A  A  M  I  V  K  V  G  E  M  M  G  F  A  F
2431 agcacacacaagcagttgataagcacgagtgattacgagttcttc
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2476 cggttatttgtctctgcggagggcgtttatggcagcttaccgcgt
     R  L  F  V  S  A  E  G  V  Y  G  S  L  P  R
2521 gtattaggcagtttatgttcagggcaatggtcgaacagcgtgaag
     V  L  G  S  L  C  S  G  Q  W  S  N  S  V  K
2566 gctaagtttatagatccagcttctaaactaaattccgttgttgag
     A  K  F  I  D  P  A  S  K  L  N  S  V  V  E
2611 atggctaggaaggcggctcgccgggcgaaaggcaatatcacgttc
     M  A  R  K  A  A  R  R  A  K  G  N  I  T  F
2656 cttgagaagttgtgcaatgcagcatttagaaaatgggccactttc
     L  E  K  L  C  N  A  A  F  R  K  W  A  T  F
2701 ggcgagcatgagcttgttgacggttacgtacatggcccacggaat
     G  E  H  E  L  V  D  G  Y  V  H  G  P  R  N
2746 aagggaggattaggagtaccgatggctgacgggagtatatacgat
     K  G  G  L  G  V  P  M  A  D  G  S  I  Y  D
2791 atagagccaataccagtggatgatacgcctccggtggagttattg
     I  E  P  I  P  V  D  D  T  P  P  V  E  L  L
2836 ggcttaccggactcagcgagccgagtagtggcaaaagagcaaata
     G  L  P  D  S  A  S  R  V  V  A  K  E  Q  I
2881 agcgacgctaaaggtatagtcggtgaatcgggtgtggtggcagag
     S  D  A  K  G  I  V  G  E  S  G  V  V  A  E
2926 gatcaattggcacaaaagatggcagggcaggtgttccgtggcaat
     D  Q  L  A  Q  K  M  A  G  Q  V  F  R  G  N
2971 atagctgcgatggaaggtgcaatggttggccaaatattaagcgat
     I  A  A  M  E  G  A  M  V  G  Q  I  L  S  D
3016 gcgcgagtgccgcctactgtagtgaacatcacgggtgtaaaatca
     A  R  V  P  P  T  V  V  N  I  T  G  V  K  S
3061 ataagacgcgagcattacgataggcatgggcatgatgtgcacgtg
     I  R  R  E  H  Y  D  R  H  G  H  D  V  H  V
3106 ttcaggcgagattacgctcggttaaagcacaggaccgacgcgctt
     F  R  R  D  Y  A  R  L  K  H  R  T  D  A  L
3151 aaaaacgctggcgcacgatacgacgcattggcagcagcggtgaag
     K  N  A  G  A  R  Y  D  A  L  A  A  A  V  K
3196 cctggttacagaagacgattggcacatgttgtaggcctcacatat
     P  G  Y  R  R  R  L  A  H  V  V  G  L  T  Y
3241 gcggttgatggtgatctactatactactggaaggagaatttaact
     A  V  D  G  D  L  L  Y  Y  W  K  E  N  L  T
3286 cttttcggctgcggcacatacttactaacggaagattactacaac
     L  F  G  C  G  T  Y  L  L  T  E  D  Y  Y  N
3331 gctgtgcagttgatggctttagtgacggcaccaggctacagtgac
     A  V  Q  L  M  A  L  V  T  A  P  G  Y  S  D
3376 aatgagataagcgagcgattagcatactacgcgactgcgcttgct
     N  E  I  S  E  R  L  A  Y  Y  A  T  A  L  A
3421 aacagcaatatgatgaattactag 3444
     N  S  N  M  M  N  Y  *
```

Apéndice 3. Secuencia de nucleótidos del genoma completo de *Beauveria bassiana* RNA virus 1 (BbRV1)

> BbRV1

gaataacaagcaccgcagcctggagtttgttatactcggcgtaggctcccttggacggaa
ggccccaccatacggtatgttggttaatgaccaactgtaagaagggtcgtgctgcccccg
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ctgatacattgctatggtatagccttttggcttcctatgatgtgatgttataggacact
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ataccccgacgcagcagcgttttcaaaacttaccaccgccggtaacatatacaccctctt
ttcaatagcaatgtaagcggtccagcttcgggacccatattctgttcgccccgtaagggg
caaatctc
```

--- Codón de inicio
--- Codón de parada
--- nt compartido entre el codón de inicio y el de parada

ORF 1 – Proteína de cápsida (CP)

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444 atgtctactgtccagactaacgcgttcctttccggtgtcatagcc
    M  S  T  V  Q  T  N  A  F  L  S  G  V  I  A
489 aacgggaggggagccctcttgacggctgacaaccagttccgccgt
    N  G  R  G  A  L  L  T  A  D  N  Q  F  R  R
534 tacgcggcgaacacacgctcctccgccaccatcggcggcaatgaa
    Y  A  A  N  T  R  S  S  A  T  I  G  G  N  E
579 gacgcacgtctcgcccgtatcttttatgaggtcggacgggtccat
    D  A  R  L  A  R  I  F  Y  E  V  G  R  V  H
624 tcaactaaggccagggctctggctgcggcccccgacggactccta
    S  T  K  A  R  A  L  A  A  A  P  D  G  L  L
669 cgagtcgatgcagcttatccaaccaccggtacgttggccgaggag
    R  V  D  A  A  Y  P  T  T  G  T  L  A  E  E
714 ttcatcggtttggcaaagaagtacacgaacttcagcgccactttt
    F  I  G  L  A  K  K  Y  T  N  F  S  A  T  F
759 gagtattccagtctggctggcatagttgagcgcattgctaaagga
    E  Y  S  S  L  A  G  I  V  E  R  I  A  K  G
804 ctggcttcacagtcagtgtttggtaatgtggacaccggtgactta
    L  A  S  Q  S  V  F  G  N  V  D  T  G  D  L
849 gcggccggtcgccccataattgtaaatgcccttggtactttcgac
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     A  A  G  R  P  I  I  V  N  A  L  G  T  F  D
 894 ggtccagttaattctctgaccaacacggttttcatcccccgtctt
     G  P  V  N  S  L  T  N  T  V  F  I  P  R  L
 939 gttaactctagtgttactggagacgtattttccgttttggttcac
     V  N  S  S  V  T  G  D  V  F  S  V  L  V  H
 984 gccgccgcgggcgagggagctgcgattgctactgatttgctcgaa
     A  A  A  G  E  G  A  A  I  A  T  D  L  L  E
1029 ctggatgccgccaccagacagcccattctgaccgtcgtcgacgcc
     L  D  A  A  T  R  Q  P  I  L  T  V  V  D  A
1074 gacggtctcgctcgtgcttgtgtcgaggctctgagattgctcggc
     D  G  L  A  R  A  C  V  E  A  L  R  L  L  G
1119 accaacatgatggccagtaaccaggggcctctctttgcattggcc
     T  N  M  M  A  S  N  Q  G  P  L  F  A  L  A
1164 ctttgtcgcggtctgcatcaggttgtcactgttgtgggccacacc
     L  C  R  G  L  H  Q  V  V  T  V  V  G  H  T
1209 gacgaaggaggcattgttcgtgacctcttacgccacagcgccttc
     D  E  G  G  I  V  R  D  L  L  R  H  S  A  F
1254 ggggtgccgttcgggggatccattttagcctcgaaccttacgct
     G  V  P  F  G  G  I  H  F  S  L  E  P  Y  A
1299 ggcctccctgccctcgccaccaattctgtcccagatgtttgctct
     G  L  P  A  L  A  T  N  S  V  P  D  V  C  S
1344 tacgttgacgccctcgcgctctcgagtgcggcacttgtcgcccat
     Y  V  D  A  L  A  L  S  S  A  A  L  V  A  H
1389 gctgatccaggacagctgtatgatggcagatggtaccccaccttt
     A  D  P  G  Q  L  Y  D  G  R  W  Y  P  T  F
1434 tacagtggaacttcttctgacgatgttgaagttcggcccggcgggg
     Y  S  G  T  S  S  D  D  V  E  V  R  P  G  G
1479 aacctccctggaaccgatgacatggcccgtcgaaacaggagtcag
     N  L  P  G  T  D  D  M  A  R  R  N  R  S  Q
1524 ctcatcggtgggctgtcccgtttttccgaggtctacgtcagaggt
     L  I  G  G  L  S  R  F  S  E  V  Y  V  R  G
1569 ttggcacagctctttgcatgtcctggtgacagccgggtcgcgtcc
     L  A  Q  L  F  A  C  P  G  D  S  R  V  A  S
1614 acgttcttcaactcgagcgtggctaacatcggcgtgagtcgtcac
     T  F  F  N  S  S  V  A  N  I  G  V  S  R  H
1659 ttgcgatatgcatccgtcgcaccatacttctggatcgagcctacg
     L  R  Y  A  S  V  A  P  Y  F  W  I  E  P  T
1704 tctttaatcccccatgatttttgggtactgacgccgaggccttt
     S  L  I  P  H  D  F  L  G  T  D  A  E  A  F
1749 ggttccggtgcactggctactaaggacgttccaagaactaagggg
     G  S  G  A  L  A  T  K  D  V  P  R  T  K  G
1794 ttctttgaggactgttgggcatccggtgtaggcgacgcagccttc
     F  F  E  D  C  W  A  S  G  V  G  D  A  A  F
1839 tctgggtatcacgtcatgttgaggaaccccagatcggcctggttt
     S  G  Y  H  V  M  L  R  N  P  R  S  A  W  F
1884 tttgctcactggttgaatcacccgcgcaacggtttgggtggaaca
     F  A  H  W  L  N  H  P  R  N  G  L  G  G  T
1929 caggttcgtcagttggacccgaacgcgatcatccacccaggtgga
     Q  V  R  Q  L  D  P  N  A  I  I  H  P  G  G
1974 catgccacgttgcctgacatcagggaccgcgttgagatggcattg
     H  A  T  L  P  D  I  R  D  R  V  E  M  A  L
2019 ccatggactgattacctctggactcggggtcagtcacccttcaat
     P  W  T  D  Y  L  W  T  R  G  Q  S  P  F  N
2064 gctccgggggaattcctgaatttagcgggcactgctggtttcatg
     A  P  G  E  F  L  N  L  A  G  T  A  G  F  M
2109 gtgaaccattacactttcgacgaggacggtatccctcagttagaa
     V  N  H  Y  T  F  D  E  D  G  I  P  Q  L  E
2154 cacctcccgacggctagagagttcgcgagcggagaggtgacaatc
     H  L  P  T  A  R  E  F  A  S  G  E  V  T  I
2199 tccgtcggccgtccgcagggtctcgctaatgggccttcgaattgg
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     S  V  G  R  P  Q  G  L  A  N  G  P  S  N  W
2244 ggtgacagtaacgcacgtcgtgcacgcactcgagctacgcgcgaa
     G  D  S  N  A  R  R  A  R  T  R  A  T  R  E
2289 ctcgccgccagtgccgcccgtgtacgggccttcggacgtccagat
     L  A  A  S  A  A  R  V  R  A  F  G  R  P  D
2334 gtcgctgagatgcccatcttgacgactgcacctcagccacgcggc
     V  A  E  M  P  I  L  T  T  A  P  Q  P  R  G
2379 tctagggtgagaccagagcctgcgagagcaaatgaccaggctgga
     S  R  V  R  P  E  P  A  R  A  N  D  Q  A  G
2424 gtcggcggttggcgtagggcttcaaactctgctgggtccggtgag
     V  G  G  W  R  R  A  S  N  S  A  G  S  G  E
2469 gctgtacctgaaccatttggtgtcccacgggatgttgtgcctcaa
     A  V  P  E  P  F  G  V  P  R  D  V  V  P  Q
2514 caccaggctgtccgttaccctgtattggctcgcaacctaggtgcc
     H  Q  A  V  R  Y  P  V  L  A  R  N  L  G  A
2559 ggaggtggcgtagcccacattcctcctccgaatcgcgggccagaa
     G  G  G  V  A  H  I  P  P  P  N  R  G  P  E
2604 ggtggcgagaatgttgaccctgtcgcaattgccggtgctgcccca
     G  G  E  N  V  D  P  V  A  I  A  G  A  A  P
2649 cccggagggccgaaccccgagtaa 2672
     P  G  G  P  N  P  E  *
```

ORF 2 – RNA polimerasa dependiente de RNA (RdRp)

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2672 atggcgaccgtcacttcgcccgacaccttcggaaagattggaata
     M  A  T  V  T  S  P  D  T  F  G  K  I  G  I
2717 tatctttccgatttactccagcggtatgggacaaataatctgccg
     Y  L  S  D  L  L  Q  R  Y  G  T  N  N  L  P
2762 agaggtggaacgtttgtctcaagattgatcactctgcagaactcc
     R  G  G  T  F  V  S  R  L  I  T  L  Q  N  S
2807 ttttcggcactccggcacgcgcatcccttgttaccagcggctgca
     F  S  A  L  R  H  A  H  P  L  L  P  A  A  A
2852 aatttgttattacttgattttccactccagactgatatcggactc
     N  L  L  L  L  D  F  P  L  Q  T  D  I  G  L
2897 cacgatttcatcgctctggtacgggaggcacactcgctgcctccg
     H  D  F  I  A  L  V  R  E  A  H  S  L  P  P
2942 atgtttgattctttgtgtatttctctatttccccctcaacctggc
     M  F  D  S  L  C  I  S  L  F  P  P  Q  P  G
2987 gagttggtcgacgtcacgcacggtcgcttagtcagaaggcttgtc
     E  L  V  D  V  T  H  G  R  L  V  R  R  L  V
3032 cggtcgtctgagttgcgggaccgtctctttcctccaaagcgtctc
     R  S  S  E  L  R  D  R  L  F  P  P  K  R  L
3077 atagcgggggaaactaagacgaatgtgacgctcggaggatgtctt
     I  A  G  E  T  K  T  N  V  T  L  G  G  C  L
3122 agatcggcccaacgactcctgggctcccacaagacggccctcatc
     R  S  A  Q  R  L  L  G  S  H  K  T  A  L  I
3167 gcccgagcgtgtatcggcttaccagccgatcatctatgcggtgtt
     A  R  A  C  I  G  L  P  A  D  H  L  C  G  V
3212 ctcattttcttattctgcgcctggcggaaactaggcgaaggagca
     L  I  F  L  F  C  A  W  R  K  L  G  E  G  A
3257 ctcggtgtggcactcttcttgtcgtcccacccaaccgagggtaag
     L  G  V  A  L  F  L  S  S  H  P  T  E  G  K
3302 tacgcgtcaatggtgttgaaggctcttggcctcaattctacagat
     Y  A  S  M  V  L  K  A  L  G  L  N  S  T  D
3347 tggggtgccctgttttgtgaaacccagtgtttggccggacgtgca
     W  G  A  L  F  C  E  T  Q  C  L  A  G  R  A
3392 acaggaacggttgatgtgaaggcggaggctcgccgtcgctgtgac
     T  G  T  V  D  V  K  A  E  A  R  R  R  C  D
3437 ccttcaaagttgaccggtgagctcattgatgttgacccagacaca
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     P  S  K  L  T  G  E  L  I  D  V  D  P  D  T
3482 ctgagggaccatgtcaggtcgatactaagggttgagctccctaat
     L  R  D  H  V  R  S  I  L  R  V  E  L  P  N
3527 gggtgtactgtacctaccctggacgatttctggacctctcgatgg
     G  C  T  V  P  T  L  D  D  F  W  T  S  R  W
3572 ttatggtgcgtcaacgggtctcacactggcgccagctccgacctt
     L  W  C  V  N  G  S  H  T  G  A  S  S  D  L
3617 ctcggcataccccgtgatttcctgtccgcgacccacgagcgggtg
     L  G  I  P  R  D  F  L  S  A  T  H  E  R  V
3662 tacagacgtgcggcttcggagaccgtcaaactagagcctctcacg
     Y  R  R  A  A  S  E  T  V  K  L  E  P  L  T
3707 tcctgggacgggtacacctctgtttccgcgagtcagaagctagaa
     S  W  D  G  Y  T  S  V  S  A  S  Q  K  L  E
3752 cacggtaagacccgtgcaatcttcgcttgtgacaccaggagttac
     H  G  K  T  R  A  I  F  A  C  D  T  R  S  Y
3797 ttcgccttctcgtgggtcttgggttctgttcagaaggcctggcgc
     F  A  F  S  W  V  L  G  S  V  Q  K  A  W  R
3842 aacagccgggtgatcctagatcccggaacaggaggacacctgggc
     N  S  R  V  I  L  D  P  G  T  G  G  H  L  G
3887 atggctcagcgcatcatcaacgctcagcgcggtggcggagtgaac
     M  A  Q  R  I  I  N  A  Q  R  G  G  G  V  N
3932 ctgatgctcgattatgatgacttcaattcccaccattcaaacggt
     L  M  L  D  Y  D  D  F  N  S  H  H  S  N  G
3977 gtcatggccatggttttcgacgaactctgcaaacacgtcggcatg
     V  M  A  M  V  F  D  E  L  C  K  H  V  G  M
4022 cccgactggtatcgggatgtgttagtcaaaagctttgacaggatc
     P  D  W  Y  R  D  V  L  V  K  S  F  D  R  I
4067 tattatactgacaataatggccgacataagattgcgggcacctta
     Y  Y  T  D  N  N  G  R  H  K  I  A  G  T  L
4112 atgagcgggcaccgagcaactacgttcatcaacagcgtcctaaac
     M  S  G  H  R  A  T  T  F  I  N  S  V  L  N
4157 gcggcctacattcgtgcggccattggcagcggccggtttgattcc
     A  A  Y  I  R  A  A  I  G  S  G  R  F  D  S
4202 ctgctctcgctccacaccggtgatgatgtgtacatacggtgtaac
     L  L  S  L  H  T  G  D  D  V  Y  I  R  C  N
4247 accctggccgactgcgcccaaatactggaggccacgaccgcctac
     T  L  A  D  C  A  Q  I  L  E  A  T  T  A  Y
4292 ggttgtaggatgaacccggcaaagcagtcaatcgggtttaggagc
     G  C  R  M  N  P  A  K  Q  S  I  G  F  R  S
4337 gctgagttcttacggatgggtattcgcggagacaaggcctacggc
     A  E  F  L  R  M  G  I  R  G  D  K  A  Y  G
4382 tatttgtcccggtcgatctcctcactagttagtgggaactggtcc
     Y  L  S  R  S  I  S  S  L  V  S  G  N  W  S
4427 tccaatgacccccctcgccccacttgagtccctacagaccctcatc
     S  N  D  P  L  A  P  L  E  S  L  Q  T  L  I
4472 acaggctgtagggctgttataaaccgttcgggagtgattgatgtg
     T  G  C  R  A  V  I  N  R  S  G  V  I  D  V
4517 gcggctttccttgcgccggcacttcgttacccgcctcaacaaatc
     A  A  F  L  A  P  A  L  R  Y  P  P  Q  Q  I
4562 tcaaaccgaacacttatcgagctacttcgaggggaagtggctctt
     S  N  R  T  L  I  E  L  L  R  G  E  V  A  L
4607 gaaggaagcccagtcttcaacacccaaggccgtatacagaattat
     E  G  S  P  V  F  N  T  Q  G  R  I  Q  N  Y
4652 gctgcgtacgtcccccgggctgatgagcttcctattccctcttca
     A  A  Y  V  P  R  A  D  E  L  P  I  P  S  S
4697 tggaaaaggcatgcgacgacggattacttgtcgtaccatgtctcc
     W  K  R  H  A  T  T  D  Y  L  S  Y  H  V  S
4742 ccaattgaggcggccgccttagagtggtcgggagcagacgctccg
     P  I  E  A  A  A  L  E  W  S  G  A  D  A  P
4787 tccctacttattgcctctagctattcaaaagggctaaataaggtc
```

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     S  L  L  I  A  S  S  Y  S  K  G  L  N  K  V
4832 ggggcggcgccgctgcctccggtttcgtttaagcggctgccggtt
     G  A  A  P  L  P  P  V  S  F  K  R  L  P  V
4877 aagcacgcacgagggtacgtgtgtgccacggatctatcgaagagg
     K  H  A  R  G  Y  V  C  A  T  D  L  S  K  R
4922 gatgttaaccccggggttttgaccaagtaccccgtaatcaaccta
     D  V  N  P  G  V  L  T  K  Y  P  V  I  N  L
4967 gtcaagtctcgactgacaaccgaggccatacttgatctactggtt
     V  K  S  R  L  T  T  E  A  I  L  D  L  L  V
5012 gtagaacttggataccgtccaagtggcgatcctcgtgagatcgcc
     V  E  L  G  Y  R  P  S  G  D  P  R  E  I  A
5057 tttggcggcgaagctgagagcaagtgtatattcggcacactatca
     F  G  G  E  A  E  S  K  C  I  F  G  T  L  S
5102 taccccgacgcagcagcgttttcaaaacttaccaccgccggtaac
     Y  P  D  A  A  A  F  S  K  L  T  T  A  G  N
5147 atatacaccctcttttcaatagcaatgtaa 5176
     I  Y  T  L  F  S  I  A  M  *
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